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VOL. 30, NO. 1 MARCH 1953

SIMULTANEOUS DETERMINATION OF SODIUM AND POTASSIUM IN SMALL VOLUMES OF FLUID BY FLAME PHOTOMETRY

BY J. A. RAMSAY, R. H. J. BROWN AND S. W. H. W. FALLOON

The Zoological and Cavendish Laboratories,
University of Cambridge

(*Received* 29 *May* 1952)

(With Plate 1)

INTRODUCTION

In two earlier papers (Ramsay, 1950; Ramsay, Falloon & Machin, 1951) a method was described by which it was possible to determine the concentration of sodium in samples of fluid of the order of 10^{-3} cu.mm. in volume. This method has now been extended to include the determination of potassium simultaneously upon the same sample. It is still in the experimental stage of development; we are fully conscious of its shortcomings and have in mind various possibilities for its improvement. Nevertheless, it is being used in its present form for biological work, some of which is already published (Ramsay, 1952), and it is therefore necessary that some description of it should be given.

The general principles and technique of flame photometry are becoming well known; various instruments have been described and some are on the market. In the majority of these instruments the solution to be investigated is atomized into the flame and the steady level of emission is measured. The method now under discussion differs mainly in that the whole of the sample is passed into the flame and the total emission is integrated. A small volume—of the order of 10^{-3} cu.mm.—of the fluid to be investigated is drawn up into a silica capillary pipette to a mark. This sample is then transferred to a platinum wire and dried. The platinum wire is thrust into the flame by a mechanical device. The emission from the flame enters a spectrometer; the spectrum falls upon a mask having two slits, one passing the sodium doublet (5890–96A.) and the other passing the potassium doublet (7665–99A.), and these emissions are allowed to reach two photomultiplier tubes. The photomultipliers are connected to integrating circuits with meter presentation. The apparatus is calibrated with known solutions drawn up to the same mark in the capillary pipette.

In essence, this is the same as the method previously described, differing only in the provision of a second channel for potassium. But for operational reasons further complications become necessary. In the earlier method the meter was first brought to rest near zero and the sample was passed into the flame; the meter made a rapid excursion and then fell back slowly. The operator noted the maximum deflexion, subtracted the zero reading and recorded the net deflexion. This was entirely practicable when only one meter had to be read, but obviously would not be practicable with two meters. The apparatus therefore had to be modified so that the meters should always start from a known zero and maintain their maximum deflexions long enough for these to be recorded. In view of the number of adjustments involved it was considered desirable to make the whole sequence of operations semi-automatic. Various other improvements were made at the same time.

MANIPULATION OF THE SAMPLE

The processes of drawing up the sample, transferring it to a storage tube, placing it upon the platinum wire and drying it are fully described in the first of the two earlier papers (Ramsay, 1950) and are still the same. Various silica capillary pipettes have been used, of volumes ranging from 0·28 to $3{\cdot}8 \times 10^{-3}$ cu.mm. It has been found useful to wash out the pipette with absolute alcohol after it has been out of use for some time and after cleaning fluids have been applied to it; this treatment with alcohol facilitates the displacement of the liquid paraffin by the aqueous solution at the surface of the silica.

APPARATUS

The burner is the same as that described by Ramsay *et al.* (1951), but modifications have been made in its supply system. In order to eliminate variations in pressure of the gas and air mains the burner was originally supplied from gasometers of sufficient capacity to maintain the flame for 20 sec. when isolated from the mains. In the new arrangement the flame burns continuously and the gasometers are connected to the mains through electrically controlled valves which allow them to fill and empty between certain limits. Variations in the quality of the town gas have been a source of trouble, and latterly 'Calor' gas has been used.

The device for carrying the platinum wire into the flame has been completely re-designed. The wire is now carried forwards into the flame by a piston driven by compressed air and returns under the action of a spring. The compressed air is admitted through an electrically controlled valve, and between this and the cylinder there is a second valve which can be adjusted to retard the flow of air and so to slow down the movements of the piston. This is important because violent accelerations can cause distortion of the wire while it is hot. The wire and its operating mechanism are mounted upon a carriage which can be swung away from the position under the entrance slit to a position which is convenient for loading the wire (see Pl. 1, B and C).

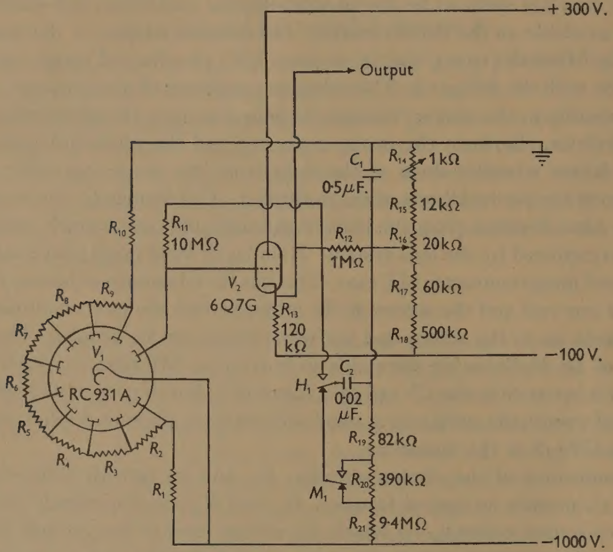
The spectrometer has the same lens system and prism, but ethyl cinnamate is now used in the latter instead of carbon disulphide. The entrance slit is adjustable in width and is ½ in. in height instead of 1 in. as before. Inevitably the arrangements of the mask are very different. The two curved slits in the mask are formed by the gaps between four movable vanes, and these are under the control of a single adjustment whereby the widths of the two slits can be varied together while each slit remains centred upon its appropriate spectral line. In addition, the height of each slit can be varied independently by a second set of vanes working at right angles to the first set. Immediately behind the mask are two mirrors set at 60°, directing the sodium and potassium emissions to their respective photomultipliers. For the details of the design and construction of the spectrometer we are indebted to Mr J. Barker of the Cambridge University Engineering Laboratory.

The sodium emission falls directly upon the photocathode of an RC931A photomultiplier. Unfortunately this tube is not sensitive to the red emission of potassium and there does not seem to be any photomultiplier combining red-sensitivity and high gain available on the British market. The solution adopted is the same as that adopted by Mitchell (1950), that is, to use a CV148 infra-red image converter in conjunction with the RC931A. The substage condenser of a microscope, placed as close as possible to the mirror, focuses the emission upon the photocathode of the image converter. Between the image converter and the photomultiplier are two bulls-eye lenses whereby some of the light from the image converter screen is focused upon the photocathode of the multiplier. Unfortunately, the screen of the CV148 is some distance from the back of the tube and only a small proportion of the light is captured by the lens system. Holliday & Wild (1951) have made use of the infra-red image converter ME1202. This has the advantage of having the photocathode at one end and the screen at the other, so that the photomultiplier can be brought right up to the screen and the worst losses can be avoided. Through the kindness of Dr Holliday we were able to borrow an ME1202 converter for trial. We found it better than the CV148 by a factor of 5, but in view of its high cost and in hopes of eventually obtaining a good red-sensitive photomultiplier we decided to use the CV148 in the meantime.

The connexions of the photomultiplier, V_1, and its cathode follower, V_2 (see Text-fig. 1), present no special features. R_{14} and R_{16} are respectively the fine and coarse zero-setting controls, by which the sitting level of the cathode follower is adjusted to give zero input to the integrator, i.e. so that the meter is steady but not necessarily exactly at zero on the scale. The only complication is the test circuit. C_2 is a condenser which is charged from the resistance chain R_{19}, R_{20}, R_{21}, and can be discharged through R_{12} by operation of relay contact H_1, thus applying a negative pulse to V_2. Operation of relay contact M_1 allows of C_2 being charged to two different voltages which are appropriate to circumstances when the amplifier V_3 (Text-fig. 2) is or is not in circuit, according to whether or not relay M is operated. This amplifier is used in the potassium channel only.

The valves V_4 and V_5 (Text-fig. 2) and their connexions form the main integrator. The input passes via relay contact L_1. The closure of relay contact E_1 reduces the time constant of the circuit, allowing C_3 to discharge quickly between determinations. The output of the integrator is connected via the meters either to a reference voltage provided by the resistance chain R_{45}, R_{46}, or, by the operation of relay contact F_1, to the output of V_6 which is known as the 'follower'.

V_6 is connected as an integrator, but it is the property of maintaining a constant anode voltage with the grid disconnected (relay contact K_1) rather than the ability to integrate which is made use of. C_4 is connected to a pair of change-over contacts which are driven by a motor and operate at about 50 times per sec. C_4 is thus charged by what is approximately the grid-anode voltage difference of the integrator and is then applied between grid and anode of the follower. In this way the grid-anode voltage difference is brought to the same value for both systems, but the actual voltages of the two anodes are not necessarily identical. To bring this difference in anode voltages under control the grid of the follower is returned

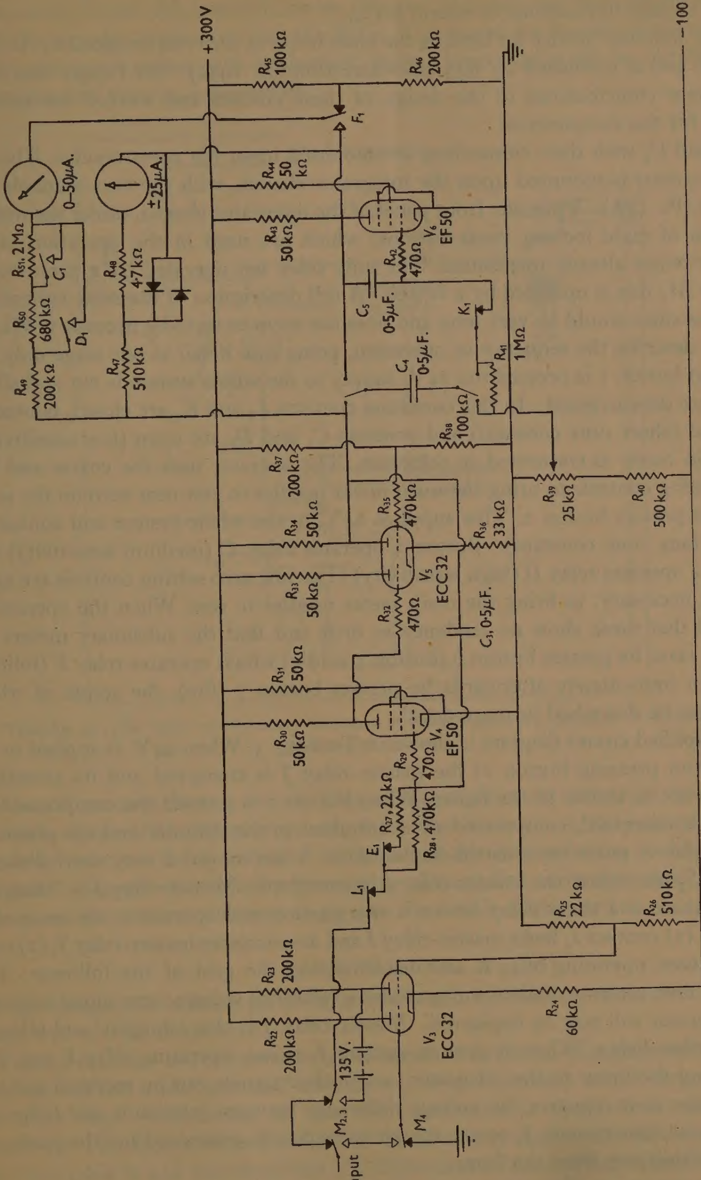


Text-fig. 1. Photomultiplier and cathode follower, zero-setting controls and test circuit. R_1–R_{10}, all 120 kΩ.

through R_{41} to a potential slightly below earth on the potentiometer R_{39}; by adjustment of R_{39}, the main meter can be set exactly to zero on the scale.

The main meter is a 0–50 μamp. instrument with a wide scale which can be read to 0·1 μamp. R_{49}, R_{50}, R_{51} and relay contacts C_1 and D_1 provide three sensitivity ranges. While the main meter can be connected either between integrator and reference voltage or between integrator and follower, the subsidiary meter is permanently connected between integrator and follower. It is a ±25 μamp. instrument, and by means of the associated resistance-rectifier network its response is made logarithmic; that is to say, it is fully sensitive to slight differences between integrator and follower voltages, and is at the same time protected from damage by large differences in voltage. The purpose of the subsidiary meter is to safeguard the main meter; it enables the operator to see that the follower is following correctly before he transfers the main meter from the reference voltage to the follower by relay contacts F_1.

The −100 V. supply is from a battery. The +4000 V. (for the CV148), the



Text-fig. 2. Amplifier, integrator and follower. Contacts as when button 1 is pressed.

+300 V. and the −1000 V. supplies are provided by power packs stabilized against mains voltage fluctuations to within 0·1 %.

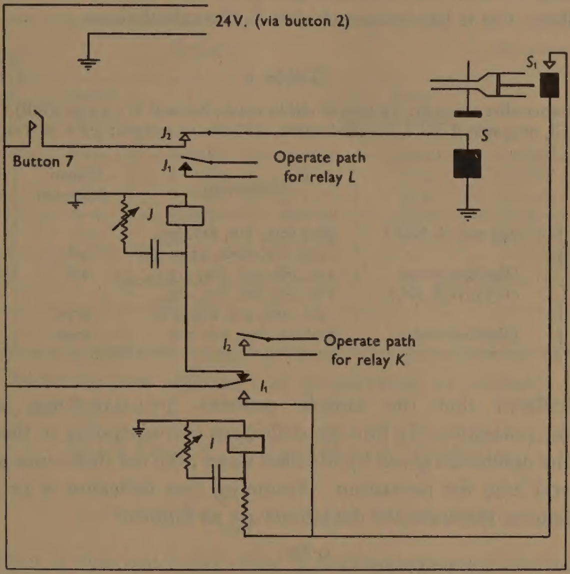
The 'follower' device for holding the main meter at zero was designed by Mr J. A. Popple and is published by him elsewhere (Popple, 1952). Mr Popple also made important contributions to the design of these circuits and worked out suitable values for the components.

V_1 and V_2 with their connexions are mounted upon the spectrometer. The rest of the circuit is mounted upon the integrator chassis, with the two channels side by side (Pl. 1, A). Upon the front panel of the integrator chassis, under the meters, is a row of eight locking press-buttons, which are used in the operation of the various relays already mentioned. The only relay not operated by a press-button is relay M; this is operated by a switch. A full description of the relay system and its connexions would be very long and does not seem to us to be necessary. We will simply describe the sequence of operation, going into detail at one stage only.

When button 1 is pressed the 24 V. supply to the whole system is cut and all the relays are de-energized. In this condition contacts L_1 and K_1 are closed, contact E_1 is closed (short time constant) and contacts C_1 and D_1 are open (low sensitivity); the main meter is connected to reference. The operator uses the coarse and fine zero-setting controls to bring the main meter needles to rest near zero on the scale. He then presses button 2. This supplies 24 V. to the whole system and contact E_1 opens (long time constant). Button 3 operates relay C (medium sensitivity) and button 4 operates relay D (high sensitivity). The fine zero setting controls are again used, if necessary, to bring the main meter needles to rest. When the operator is satisfied that these show no tendency to drift and that the subsidiary meters are reading zero, he presses button 6 (button 5 is idle) which operates relay F (follow), and then immediately afterwards he presses button 7 (fire), the action of which must now be described in more detail.

A simplified circuit diagram is shown in Text-fig. 3. When 24 V. is applied to the system (on pressing button 2) the master-relay J is energized and its associated contacts are as shown in the figure. When button 7 is pressed the compressed air valve S is energized, compressed air is admitted to the cylinder and the platinum wire begins to move towards the flame. After it has moved a very short distance contact S_1 closes and the master-relay I is energized. Master-relay I is 'slugged' so that there is a short delay between energization and operation. As soon as it operates (1) contact I_1 locks master-relay I and de-energizes master-relay J, (2) contact I_2 closes, operating relay K and disconnecting the grid of the follower. The follower now ceases to follow and provides a reference voltage; any signal entering the integrator will now be registered. Master-relay J is also 'slugged' and releases after further delay. When it does so contact J_1 closes, operating relay L and disconnecting the input to the integrator; no further signals can be received and the main meter now registers the voltage difference between integrator and follower. At the same time contact J_2 opens, the air valve S is de-energized and the platinum wire is withdrawn from the flame.

In this way signals from the photomultiplier are registered only during the short interval, or 'gate', between the operation of master-relay *I* and the release of master-relay *J*, and this interval can be adjusted to cover only that period during which the sample is vaporized into the flame. The reason for having master-relay *J* initially energized is that with a 'slugged' relay a longer delay can be obtained on de-energization than on energization.



Text-fig. 3. The 'gate' mechanism. Contacts as when button 2 is pressed. For further explanation see text.

Button 8 operates the test circuit. This, of course, does not come into the normal sequence of operation; when required, it replaces button 7 in the sequence. The test circuit involves the operation of the 'gate' mechanisms as before, and by means of a third 'slugged' master-relay the test signal is applied while the 'gate' is open. It does not seem to us necessary to go into the details of this circuit.

PERFORMANCE

The performance of a flame photometer is affected by a variety of factors, some of which will now be considered.

A useful measure of performance is the standard deviation of a series of observations carried out under identical conditions, using a quantity of the element near the lower limit of detection so that the instrument is operated at maximum sensitivity. The observations recorded in Table 1 were made with this in view.

In this table it will be noted that the deflexions in the sodium channel due to distilled water are considerable; this is partly due to the presence of sodium in the distilled water—not that in the measured sample, which is of course negligibly small, but that in the droplet (about 0·5 cu.mm.) into which the sample is ejected from the pipette. These droplets when deposited directly upon the platinum wire give an average deflexion of 2·5 divisions. There must be additional contamination with sodium during the manipulation and storage of the sample, but it is not yet known how this occurs. One of the deflexions in the distilled water series appears to be anomalous, but it has been included in the calculations.

Table 1

Width of entrance slit: 0·012 in. Height of slit in mask, Na and K: 0·5 in. (full). Test solutions: 0·43 mM./l. NaCl, 0·43 mM./l. KCl, distilled water. Volume of sample: $3{\cdot}8 \times 10^{-8}$ cu.mm.

		Deflexions	Mean deflexion	S.D.
Sodium channel	0·43 mM./l. NaCl	9·7, 10·9, 8·7, 8·8, 8·3, 9·7, 9·1, 10·2, 10·9, 9·8	9·61	±0·89
	Distilled water	4·3, 3·8, 4·7, 7·9, 3·3	4·8	±1·81
Potassium channel	0·43 mM./l. KCl	1·8, 3·2, 2·6, 2·5, 2·9, 2·5, 2·6, 4·2, 2·6, 2·6	2·75	±0·62
	Distilled water	1·0, 0·3, 0·1, 0·6, 0·3	0·46	±0·41

It is calculated that the sample contains $3{\cdot}74 \times 10^{-8}$ mg. sodium and $6{\cdot}35 \times 10^{-8}$ mg. potassium. To find the deflexions corresponding to these quantities we subtract the deflexions given by distilled water; the net deflexions are then 4·81 for sodium and 2·29 for potassium. Assuming that deflexion is proportional to amount of element the standard deviations are as follows:

Sodium:

$$\pm 3{\cdot}74 \times 10^{-8} \times \frac{0{\cdot}89}{4{\cdot}81}$$

$$= \pm 6{\cdot}9 \times 10^{-9} \text{ mg.} = \pm 3{\cdot}0 \times 10^{-10} \text{ m.equiv.}$$

Potassium:

$$\pm 6{\cdot}35 \times 10^{-8} \times \frac{0{\cdot}62}{2{\cdot}29}$$

$$= \pm 1{\cdot}72 \times 10^{-8} \text{ mg.} = \pm 4{\cdot}4 \times 10^{-10} \text{ m.equiv.}$$

From these figures it is possible to calculate the smallest difference which can be established at a particular level of statistical significance with a given number of determinations. For example, if we have two solutions and make four determinations on each, the smallest difference which can be established at the $P = 0{\cdot}01$ level of significance is $7{\cdot}9 \times 10^{-10}$ m.equiv. of sodium and $10{\cdot}5 \times 10^{-10}$ m.equiv. of potassium.

The above is only true for amounts near the lower limit of detection. When greater amounts are used it is found that, as with most analytical methods, the error is roughly proportional to the amount of the element present. Opportunities for investigating this matter have presented themselves throughout the course of biological investigations in which the method has been used. In one such investigation a solution containing 171 mM./l. NaCl and 171 mM./l. KCl was used as a standard of reference, and several readings were taken on this solution during each series of observations. This data is summarized in Table 2.

Table 2

Width of entrance slit: 0·10 in. Height of slit in mask: Na, 0·15 in.; K, 0·5 in. Test solution: 171 mM./l. NaCl, 171 mM./l. KCl. Volume of sample: $0{\cdot}28 \times 10^{-3}$ cu.mm.

Series	No. of observations	Sodium			Potassium		
		Mean deflexion	S.D.	S.D. %	Mean deflexion	S.D.	S.D. %
1	7	29·3	±0·46	±1·59	40·4	±0·855	±2·12
2	8	30·6	±1·085	±3·54	39·2	±0·825	±2·11
3	9	29·0	±0·96	±3·32	33·2	±1·19	±3·58
4	9	31·7	±0·88	±2·78	33·8	±0·61	±1·80
5	7	30·5	±1·06	±3·48	32·8	±0·66	±2·02
	Total 40	Average S.D. ±2·97 %			Average S.D. ±2·36 %		

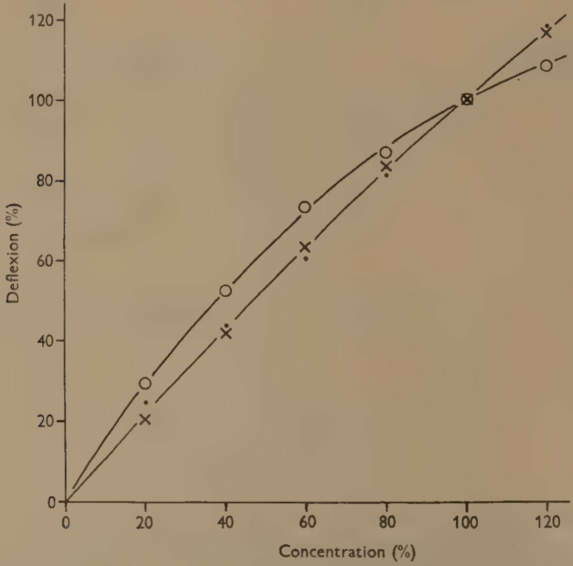
The sample is calculated to contain $4{\cdot}8 \times 10^{-8}$ m.equiv. both for sodium and for potassium. Assuming that deflexion is proportional to amount, the standard deviations are as follows:

Sodium: $$\pm 4{\cdot}8 \times 10^{-8} \times \frac{2{\cdot}97}{100} = \pm 1{\cdot}42 \times 10^{-9} \text{ m.equiv.}$$

Potassium: $$\pm 4{\cdot}8 \times 10^{-8} \times \frac{2{\cdot}36}{100} = \pm 1{\cdot}13 \times 10^{-9} \text{ m.equiv.}$$

Consideration of this and many other series of observations carried out under a variety of conditions leads to the general conclusion that when the deflexions lie in the middle range (i.e. 20–40 divisions) of the scale, the standard deviation amounts to some 3 % of the mean deflexion.

We must now examine more closely the relation between deflexion and amount of element, which has hitherto been assumed to be one of simple proportion. As will be seen from Text-fig. 4, the assumption is certainly untrue. For low concentrations there is some departure from linearity, but it is not likely to be a serious source of error. At high concentrations the departure from linearity is considerable. These results are compatible with what was found in the earlier work (Ramsay, 1950). Text-fig. 2 of that paper shows a relationship between deflexion and concentration which almost exactly fits the low-concentration curve in Text-fig. 4 of the present paper. The departure from this relationship was previously observed when the amount of sodium in the sample exceeded $5{\cdot}5 \times 10^{-6}$ mg. It is now observed at about $3{\cdot}0 \times 10^{-6}$ mg. At high concentrations the departure from linearity has the effect of reducing the accuracy of the method. For example, at a sodium concentration of 14 m.equiv./l. an error of 3 % in deflexion corresponds to an error of 3·3 % in concentration; at 140 m.equiv./l. it corresponds to an error of 5·7 % in concentration. It is therefore desirable to work with the smallest quantities which will give adequate deflexions. A difficulty frequently arises, however, when one element is present in much greater concentration than the other, as is generally the case with sodium relative to potassium in biological fluids. In order to get adequate deflexions on potassium it may be necessary to raise the amount of sodium in the sample beyond the point at which the percentage error begins to increase.



Text-fig. 4. Relation between deflexion and concentration. •: Na, 14 m.equiv./l.; ×: K, 40 m.equiv./l.; ○: Na, 140 m.equiv./l. Volume of pipette, $1{\cdot}9 \times 10^{-3}$ cu.mm. Each point is the mean of at least three determinations.

In the earlier paper the interpretation given to the relation between deflexion and concentration was that at low concentrations the emission was directly proportional to the amount of element present in the sample and that the slight non-linearity of the low-concentration curves arose in the electrical side of the apparatus. The present results are in keeping with this interpretation. The considerable departures from linearity at high concentrations are commonly experienced in other methods of flame photometry.

It is also necessary to take account of interference, i.e. the enhancement or repression of the emission of an element by the simultaneous presence of other elements or radicles. Where the concentrations of the interfering substances are known it is merely a matter of patient calibration to establish the nature and extent of the interference. But where we have to determine sodium and potassium in a solution of unknown composition the possibilities of serious error are considerable.

Fortunately in biological fluids the upper and lower limits of concentration are known with some certainty for most substances. It is therefore possible to determine by a preliminary survey the interference errors to which the method is liable for the particular fluid which is to be investigated. Purely for the purpose of illustration a short survey of interference errors has been carried out with certain substances in such concentrations as may occur in normal human urine. The substances in question and their concentrations were as follows:

Na^+	140 m.equiv./l.	Cl^-	102 m.equiv./l.	Urea	330 mM./l.
K^+	40 m.equiv./l.	$HPO_4^=$	56 m.equiv./l.		
Ca^{++}	12 m.equiv./l.	$SO_4^=$	42 m.equiv./l.		
Mg^{++}	8 m.equiv./l.				

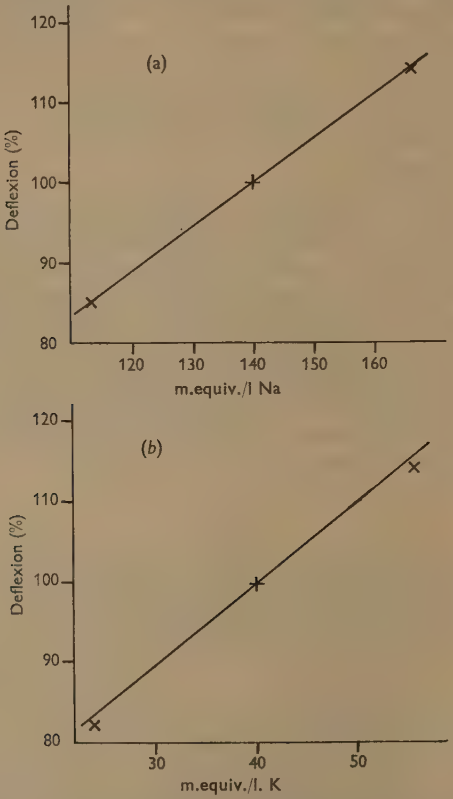
A preliminary survey, carried out in the coal gas flame, revealed considerable mutual interference between sodium and potassium. The addition of KCl in a concentration of 40 mM./l. to a solution containing 140 mM./l. NaCl reduced the sodium deflexion by 1·5 %. The addition of NaCl in a concentration of 140 mM./l. to a solution containing 40 mM./l. KCl reduced the potassium deflexion by 49 %. The effect of potassium on sodium is negligible; the effect of sodium on potassium, although very great, is not of outstanding importance since these are the substances which are being measured, and adequate calibration makes full correction possible. But the effects of other substances whose concentrations are not measured by the method are more serious. These are listed in Table 3.

Table 3

Width of entrance slit: 0·0075 in. Height of slit in mask: Na, 0·05 in.; K, 0·5 in. Volume of sample: $1{\cdot}9 \times 10^{-3}$ cu.mm. The deflexions given by sodium and potassium in the solutions tested are compared with the deflexions given by sodium and potassium in a reference solution containing 140 mM./l. NaCl × 40 mM./l. KCl. All concentrations are given in m.equiv./l. or mM./l.

Solutions tested								Interference errors (%)	
Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	$HPO_4^=$	$SO_4^=$	Urea	Na	K
140	40	12	—	192	—	—	—	− 4·0	− 2·2
140	40	—	8	188	—	—	—	− 5·0	− 4·0
140	40	12	8	200	—	—	—	− 6·2	− 4·0
140	40	—	—	124	56	—	—	+13·5	+ 6·2
140	40	—	—	138	—	42	—	+ 7·5	+ 7·0
140	40	—	—	82	56	42	—	+17·0	+25·0
140	40	—	—	180	—	—	330	− 2·8	− 1·9

The interference errors caused by phosphate were further tested using various concentrations of 'Specpure' ammonium phosphate instead of distilled water for the drop into which the sample was ejected from the pipette. The volume of the drop in these tests was approximately 1 cu.mm. It was found that the effect of ammonium phosphate became noticeable with solutions of 0·01 mM./l. and reached its maximum at 0·075 mM./l., without further significant change up to 10 mM./l. It therefore seemed possible to swamp the effect of phosphate in the sample by adding excess of ammonium phosphate in the drop. The experiments summarized in Table 3 were therefore repeated using a drop of 1 mM./l. ammonium phosphate instead of distilled water. At this stage, for other reasons, 'Calor' gas was substituted for coal gas, but it was first ascertained that phosphate interference was not substantially affected by this change.



Text-fig. 5. Calibration curves in the presence of excess ammonium phosphate. (*a*) For sodium, in the presence of 40 m.equiv./l. potassium; (*b*) for potassium, in the presence of 140 m.equiv./l. sodium.

A solution containing 140 mM./l. NaCl + 40 mM./l. KCl was used as reference and the apparatus was calibrated over the ranges ±20% of these concentrations. The calibrations are reproduced in Fig. 5, each point being the mean of at least three determinations. Over these limited ranges we are prepared to accept the relation between concentration and deflexion as linear. In view of the very serious interference effect of sodium on potassium it seemed likely that further calibration curves for potassium in the presence of different concentrations of sodium would have to be prepared. Fortunately it was found that over the ranges of concentration in question the change in this effect was less than 3% and it has been neglected.

Table 4

(Conditions as described for Table 3.)

Solutions tested								Interference errors (%)				No. of obervations	
								Deflexion		Concentration			
Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	$HPO_4^=$	$SO_4^=$	Urea	Na	K	Na	K	Reference	Test
140	40	12	—	192	—	—	—	−4·3	−3·0	−6·2	−3·75	8	4
140	40	—	8	188	—	—	—	+1·0	−1·0	+1·4	−1·25	5	4
140	40	12	8	200	—	—	—	0	−1·0	0	−1·25	6	5
140	40	—	—	124	56	—	—	0	−2·5	0	−3·1	4	4
140	40	—	—	138	—	42	—	−0·5	−2·5	−0·7	−3·1	4	4
140	40	—	—	82	56	42	—	0	−1·0	0	−1·25	6	5
140	40	—	—	180	—	—	330	−3·8	0	−5·3	0	8	4

The interference errors in the presence of excess ammonium phosphate are listed in Table 4, use being made of the calibration curves in converting deflexion error into concentration error. As compared with Table 3 a substantial improvement can be noted, and it is particularly satisfactory that the swamping effect of ammonium phosphate appears to make itself felt upon the sulphate error as well as upon the phosphate error.

Errors of the order of 6% may possibly be unacceptable in chemical work and in studies of mammalian physiology in which a high degree of accuracy is called for. In any case, the integrative method offers no advantage over the more usual non-integrative atomizing methods where the amount of fluid available is not a consideration. If the present method has anything to recommend it, it is that it can be used with the very small volumes which have to be accepted in physiological work upon invertebrates, where errors of 6% are by no means prohibitive. It is in this field that we believe it may find useful application.

DISCUSSION

The sensitivity of this method is greater than that of any other method hitherto described. For the Beckman flame spectrophotometer Gilbert, Hawes & Beckman (1950) state that 0·05 cc. of 0·01 p.p.m. sodium, which represents 2×10^{-8} m.equiv. of sodium, will give a deflexion 0·5% of full scale. From the data in Table 1 it can be calculated that a similar deflexion would be given by $8{\cdot}5 \times 10^{-11}$ m.equiv. of sodium. It would be a matter of no great difficulty further to increase the sensitivity to sodium by a factor of, say, 5. But nothing would be gained by so doing, since in practice it is almost invariably the lack of sensitivity to potassium which sets the lower limit to the volume of sample which can be used.

The consistency of the method, however, is distinctly poor by modern standards.

Our figure of ±3% of the total quantity is to be compared with corresponding figures of the order of ±0·5% reported by Weichselbaum & Varney (1949) Gilbert *et al.* (1950) and White (1952). We believe that the lack of consistency is associated with the means adopted for introducing the sample into the flame. With a platinum wire of 0·005 in. diameter the vaporization and emission are over in less than a second. Oscillograms of the pulse recorded from the cathode follower with the integrator disconnected show considerable variation in form, although taken under identical conditions.

We are not impressed with the urgency of improving the consistency of the method in view of the greater hazards to accuracy which are presented by the interference errors. This is a subject which is at the moment in some confusion. Take, for example, the mutual interference of sodium and potassium. Most observers find that the effect is one enhancement, e.g. Belke & Dierkesmann (1948), Bernstein (1950), Brealey & Ross (1951), Domingo & Klyne (1949), Knight, Mathis & Graham (1951), Riehm (1948), Smit, Alkemade & Verschure (1951), Weichselbaum & Varney (1949), West, Folse & Montgomery (1950). In the present work the effect was found to be one of repression, as was also found by Parks, Johnson & Lykken (1948). The very extensive studies made by Bills, McDonald, Niedermeier & Schwarz (1949) showed that the sign of the interference changed with dilution. Or, again, the case of interference by phosphate; in the present work phosphate has been found to enhance the emission of sodium and potassium. But Brealey & Ross (1951), Crimson (1948), Osborn & Johns (1951), Parks *et al.* (1948) found repression, while Bowman & Berliner (1949), Domingo & Klyne (1949), Riehm (1948) and Shapiro & Hoagland (1948), failed to discover any effect at all.

The only serious attempt to make a physical interpretation of interference phenomena in flame photometry is that of Smit *et al.* (1951), and their treatment is restricted to the mutual interference between sodium and potassium. It is doubtful whether our knowledge of the kinetics of reactions in flames is sufficiently advanced to promise any comprehensive theory of interference in the near future, and it seems likely that flame photometry will remain an empirical science for some time to come. We must therefore look to various empirical methods of reducing interference errors.

The device of the internal standard, introduced by Berry, Chappell & Barnes (1946), has been used with success in reducing errors due to variation in flame temperature, rate of atomization, etc. A known concentration of lithium is added to the tested solution and if the apparent concentration of lithium differs from its known concentration by a certain factor, that same factor is applied as a correction to the apparent concentrations of sodium and potassium. This method of correction is of course inapplicable to the mutual interference between sodium and potassium, as pointed out by Bernstein (1950), and it further appears that the interference effects of anions are not suffered equally by all the alkali metals (Parks *et al.* 1948; Gilbert *et al.* 1950).

Knight *et al.* (1951) make up their calibrating solutions in the form of a 'synthetic known', that is to say, the calibrating solution is made as nearly as possible identical with the tested solution except for the element involved in the calibration. This procedure should give reliable results provided that the concentrations of the interfering substances in the tested solution can be accurately foretold. A safer method is the use of 'radiation buffers' as recommended by West *et al.* (1950), who measured the sodium, potassium and calcium concentrations in river water. For the determination of sodium they added excess of KCl, $CaCl_2$ and $MgCl_2$, thereby swamping the effects of these cations in the river water itself; similarly for potassium and calcium, *mutatis mutandis*. This line of approach has also been followed by Eggertsen, Wyld & Lykken (1951) in overcoming the interference due to acids and salts, and has been used for phosphate interference as described earlier in this paper.

The strongest impression which one gains from a survey of the literature dealing with flame photometry is that each investigation has led to a special solution. This is no doubt in part a result of the unpredictable interference effects which are encountered, but it is also a consequence of the innumerable minor variations in design which have recommended themselves to different users. If a limited number of commercial types of flame photometer come to replace the variety of 'home-made' instruments now in use, it may be that the subject will gradually be brought into some sort of order. Until some general principles can be established we remain of the opinion that, even if the same instrument is used throughout, each different kind of fluid,—blood, urine, etc.,—presents a fresh problem, requiring a fresh approach, and that attempts to argue from one set of conditions to another will be unprofitable.

Apart from its usefulness in cases where only small volumes are available, the integrative method as here described avoids the difficulties of maintaining the same rate of atomization when solutions of different physical properties are compared. It does not entail the use of special glassware for very dilute solutions. It does not seem to us that the provision of a third channel for internal standard would substantially improve its accuracy. What is felt most keenly is the lack of a suitable photomultiplier for the potassium channel. A tenfold increase in the sensitivity to potassium would avoid the loss of accuracy on sodium which occurs when more than 3×10^{-6} mg. sodium is present in the sample, by making it possible for smaller samples to be used. It is encouraging to know that experimental models of high-gain photomultipliers with suitable photocathodes have been made, and at the same time exasperating to be unable to obtain one.

SUMMARY

1. A method of flame photometry is described, by which the amount of sodium and potassium in biological fluids can be determined simultaneously, using samples of the order of 10^{-3} cu.mm.

2. When the method is tested with quantities near the lower limits of detection (of the order 4×10^{-8} mg. sodium, 6×10^{-8} mg. potassium) the reproducibility, measured as standard deviation, is $\pm 7 \times 10^{-9}$ mg. sodium and $\pm 17 \times 10^{-9}$ mg. potassium.

3. When larger quantities (up to certain limits) are used, the standard deviation is approximately ±3% of the quantity in the sample, both for sodium and for potassium.

4. As is usual in flame photometry serious errors can be caused by the presence of other substances in the sample. In the case studied for purposes of illustration it is shown that these interference errors can be reduced to the order of ±6% by the addition of excess ammonium phosphate.

We wish to thank Dr R. L. Mitchell and Mr K. E. Machin for their comments upon the typescript of this paper.

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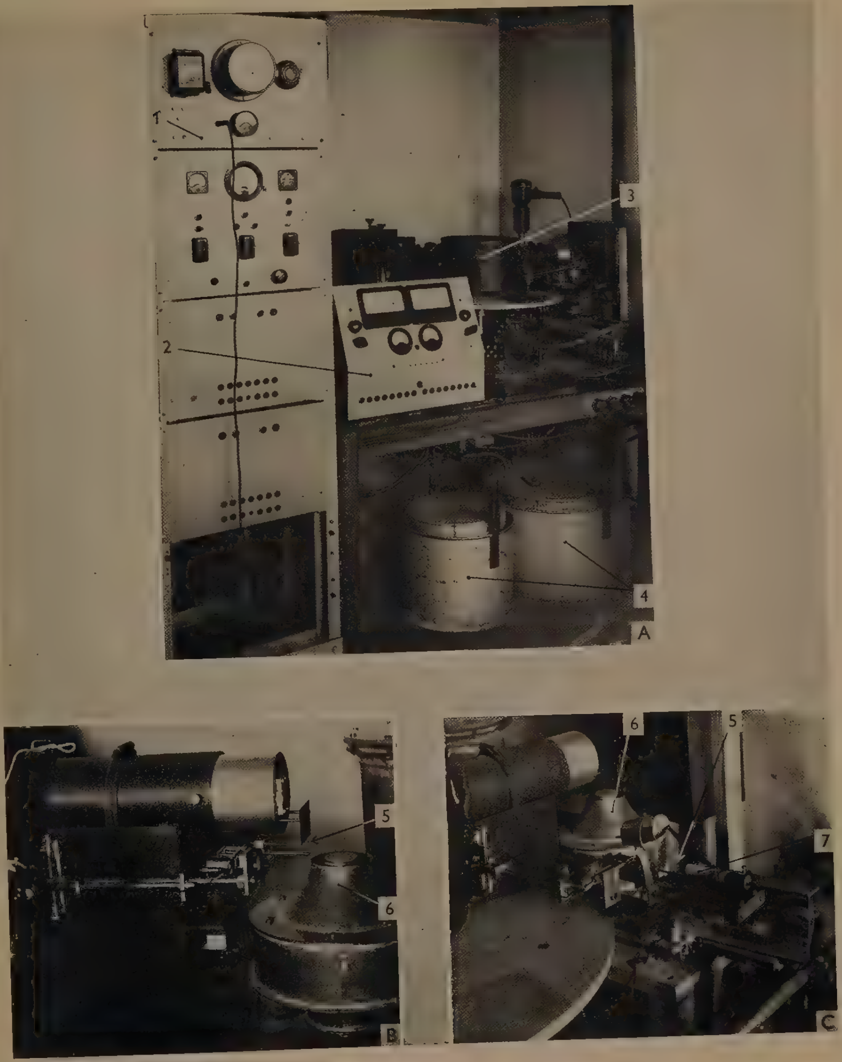

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EXPLANATION OF PLATE 1

A. General view of the apparatus.

B. Close-up, to show the platinum wire in the firing position.

C. Close-up, to show the platinum wire in the loading position.

1: rack for power supplies. 2: integrator chassis and panel; main meters (square), subsidiary meters (round), coarse and fine zero-setting controls (to right and left of meters), press-buttons (white) under subsidiary meters. 3: spectrometer; entrance slit and collimator on right, box containing photomultipliers, etc., on left. 4: gasometers. 5: platinum wire. 6: burner. 7: coil of nichrome wire enclosed in a glass tube; this slides forwards on guides so as to cover the platinum wire.

THE REGIONAL PROPERTIES OF AMPHIBIAN ORGANIZER TISSUE AFTER DISAGGREGATION OF ITS CELLS IN ALKALI

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(With Plate 2)

INTRODUCTION

The idea that regional differentiation in the embryo is governed by 'fields' or 'gradients' in the metabolism of the organizer has for some time been popular among experimental embryologists. It was a development of Child's famous 'theory of axial gradients' (Child, 1916*a*, *b*; also review, 1946), according to which antero-posterior differentiation in animals depended upon differences in the metabolic rate of the tissues. Although there are many objections to this theory, it has not so far been disproved. Substantial evidence has accumulated that certain biochemical gradients do exist in vertebrate embryos, and, although the connexion between these and morphological differentiation is unknown, it has remained convenient to use the terms 'field' and 'gradient' when describing the properties of the organizer.

Waddington & Schmidt (1933) introduced the term 'individuation field' in connexion with the pattern of antero-posterior determination in the chick, and the influence of the host on grafted tissues. They emphasized the action of the organizer as a unifying system. Any competent tissue added to the system tends to be incorporated into it so that a single normal embryo results; the region over which such a unifying activity occurs is the individuation field. Further, when an organizer is grafted into the neighbourhood of competent tissue, it may not only induce in that tissue structures which become assimilated into a unified system together with the derivatives of the organizer, but it may induce organs which remain independent of the graft. Such inductions, in so far as they are morphologically organized, are themselves 'individuated'. In many cases, particular parts of the organizer are known to induce specific types of organs, and in these the organizer must have transmitted some influence which has controlled the individuation of the induction. Waddington & Schmidt argued that this transmissible influence was connected with the individuation field by which the organizer's own morphological unity is ensured. Since any specific organ which is induced will be characteristic of some particular region of the embryo, the capacity of an organizer to transmit specific individuating influences may be referred to as its 'regionality'. It may be noted, however, that it is quite possible that within an induction individuating forces might arise which were not specifically determined by any influence transmitted by the inductor;

this would be 'self-individuation' (cf. Waddington, 1950; Lehmann, 1945, 'Selbstorganisation').

Using a more analytical approach, Dalcq (1940) attempted to explain differentiation in the amphibian embryo in terms of 'morphogenetic potentials'. The future dorsal lip region, and other tissues in relation to it, were supposed to be determined by potentials computed from their positions along two axial metabolic gradients: one dorsoventral, and the other antero-posterior. The existence of these two gradients is fairly well established, but it is questionable how far they control regional differentiation.

Since no qualitative explanation has ever been given which could explain all the known facts about regional differentiation, many leading embryologists (e.g. Dalcq, 1940; Lehmann, 1945; Nieuwkoop, 1946; Waddington, 1937; Yamada, 1950) still favour the point of view that there are quantitative biochemical gradients within the organizer, at least in the early stages of development, although these probably later give rise to qualitative differences. Waddington & Yao (1950) have paid particular attention to the space-time relationship between archenteron roof and dorsal ectoderm during gastrulation, and have given contour maps of the suggested distribution of the evocator in levels of concentration extending outwards from a maximum in the anterior mesoderm. In their work the integrative quality of organizer action is once more emphasized. It is suggested that this quality normally depends upon both the temporal and the spatial sequence of invagination.

Although little is known about the influence of time on the regional properties of the organizer, a few observations have been made on the influence of its spatial configuration. Spemann (1931*b*) noted that the success of secondary inductions depended partly on the direction and extent to which the implanted organizer had invaginated. Altekrüger (1932) claimed that the nature of inductions was determined by the external shape of the implanted tissue.

In the light of the ideas which have just been discussed, it was remarkable when Holtfreter (1944*b*) showed that the organizer can apparently induce normally after its cells have been first disaggregated in alkali and then allowed to reaggregate in random order. He found that the now chaotically arranged cells were still able to induce a normal symmetrical nervous system when implanted into an early gastrula. Such a treatment must have profoundly disturbed any individuation field which depends on the arrangement of the cells with respect to one another. Since the structures induced by such organizers are reasonably well individuated and therefore regionally specific, we have to consider how far the individuation of the disaggregated cells becomes restored, and whether the initial regional specificity of the parts of the organizer is also regained or whether the individuation of the induced structures arises autonomously.

There are, however, two points to be borne in mind in considering Holtfreter's results. One is, that in the young organizer (early gastrula dorsal lip) which he used, regional differences were not yet established. There is abundant evidence (e.g. Spemann, 1931*b*; Holtfreter, 1933*a*; Hall, 1937; Waddington & Yao, 1950) that the regional properties of the organizer are modifiable at this stage. Secondly, it could be suggested that the symmetry of the nervous tissue induced by reaggregated organizer was imposed by the host's individuation field.

The following experiments were designed to test these two possibilities, by repeating Holtfreter's procedure, but this time using organizer tissue from the late gastrula (Harrison's stage 12–12½) and explanting it into early gastrula ectoderm in order to avoid any possible influence from a host embryo. In addition, a few explants and implants of early gastrula dorsal lip were carried out to confirm Holtfreter's results.

Yamada (1937) and Waddington & Yao (1950) have presented evidence that regional differentiation in the archenteron roof of the late gastrula is still labile. On the other hand, Mangold (1929*a*) and ter Horst (1948) were able to detect regional differences of a quantitative nature in the archenteron roof of the early neurula. In the present work it was found that the completed gastrula (H. 12½), was the latest stage at which it was possible successfully to separate the archenteron roof from the dorsal ectoderm. Okada & Hama (1945) appear to be the only workers who have studied the regional properties of the organizer at this stage, using explants. But the differences which they noticed were in the types of sense organs induced: they did not describe any tissue recognizable as brain or neural tube. Since sense organs are secondary inductions, they do not seem a sound criterion for recognition of regional differences in the primary inductor. In the following work, therefore, an extensive series of control explants was carried out (series 4 (*a*) and (*b*)) in order to establish the existence of regionality in the normal primary organizer, before studying the effects of disaggregation of its cells.

MATERIAL AND METHODS

The species used for these experiments was *Triturus alpestris*, obtained from sources in Belgium and Switzerland, and kept in laboratory aquaria. Their normal breeding season was prolonged by injecting females with Pregnyl (Organon Laboratories Ltd.).

The embryos were decapsulated, washed and operated on in Holtfreter's saline (Holtfreter, 1931) containing a phosphate buffer instead of the usual sodium bicarbonate buffer. To every 50 ml. of Holtfreter saline, 1 ml. of the following mixture of phosphates was added: 1 part 2·3 % KH_2PO_4, to 3 parts 1·5 % K_2HPO_4 solution. This gave a constant pH of 7·0, as measured by an electrolytic pH meter. It proved more satisfactory than sodium bicarbonate which cannot be sterilized in solution and gives a pH nearer 8·0, which is not so suitable for explants.

Explants were cultured in full-strength Holtfreter saline, and embryos in the saline diluted to one-tenth, in dishes floored with a layer of 2 % agar.

In spite of precautions to maintain asepsis (see Hamburger, 1942), over 60 % mortality occurred in earlier experiments. So in the latter half of the 1950 season sodium sulphadiazine (May & Baker) was used as a bactericidal agent. Detwiler, Copenhaver & Robinson (1947) recommend an 0·5 % solution of this drug in Holtfreter saline, but it was found in the present work that any attempt to buffer the solution at this concentration resulted in a precipitate of sodium hydrogen phosphate and a rise in pH to 8·2. Finally it was found satisfactory to use the sodium sulphadiazine at 0·1 % in the buffered Holtfreter saline, adding in addition to the mixed phosphate buffer, 1 ml. of M/15 KH_2PO_4 to every 15 ml. of saline. This gave a pH of 6·85. Crystals of sodium hydrogen phosphate formed slowly, but this had no adverse effect on the cultured tissues. Over 90 % of explants survived in this solution, for up to 14 days.

Operations were carried out with fine, two-edged mounted steel needles, the embryo resting in a depression on the floor of a waxed solid watch-glass. Presumptive ectoderm was taken from the early gastrula at Harrison's stage 10, except in series 6 where ventral ectoderm from a completed gastrula (Harrison's stage 12–12½) was used. As organizer, the complete dorsal lip from the early gastrula (series 2, 5 and 6), or the archenteron roof mesoderm from the late gastrula (series 3 and 4) was used.

To disaggregate the organizer, it was placed in Holtfreter saline containing 0·7 g./l. of potassium hydroxide. The pH of this solution had been adjusted to 10·0 by addition of 1 ml. of borax-soda buffer solution (Reilly & Rae, 1943), to every 50 ml. of saline. After 2–5 min., varying with the size of the piece of tissue, the cells had rounded off and fallen apart. Further exposure to the alkali resulted in their complete disintegration. The disaggregation process was watched under the binocular, and as soon as it was complete, the loose cells were transferred in a very fine pipette, with as little of the alkali as possible, to a solid watch glass containing normal Holtfreter saline and floored with 2 % agar. The cells were collected together into a heap with the help of needles, and left undisturbed for 2 hr. or more. In this time they reaggregated into a sheet or ball of tissue which could either be explanted into ectoderm or implanted into another gastrula. The explants or implants were left undisturbed in the watch-glasses overnight, then transferred to culture dishes.

Explants were cultured for 9–14 days, and implants for 6–9 days. They were observed daily, disturbing them as little as possible. They were finally fixed in Bouin's fixative, dehydrated through 50, 70 and 95 % alcohols, cleared in methyl benzoate, and taken via benzene to paraffin wax of 52° C. melting-point. Sections were cut at 10 μ, and stained with Ehrlich's or with Delafield's haematoxylin.

EXPERIMENTS

SERIES 1. CONTROL EXPLANTS OF EARLY GASTRULA ECTODERM WITHOUT ORGANIZER TISSUE

Barth (1941) and Holtfreter (1944*a*, 1945, 1947) found that the early gastrula ectoderm of *Amblystoma punctatum* and *Triturus torosus* was capable of differentiating into neural tissue even in the absence of an organizer, if the pH of the culture solution was suitably adjusted. Since the saline used in the present experiments had been modified both in pH and salt content by the addition of phosphate buffers and of sodium sulphadiazine, a control series of explants of early gastrula ectoderm alone was carried out to verify that neuralation could not occur under these conditions.

Twenty-one explants of early gastrula ectoderm were cultured for 12 days in the same saline as was used throughout the experiments. After this time four explants had disintegrated, and the remaining seventeen were irregularly shaped, with folds on their surface. In histological sections, no neural tissue was seen.

It was clear that the ectoderm had no power to self-differentiate into neural tissue, and that neither the modified saline nor the sodium sulphadiazine acted as evocators. All neural structures obtained in the following experiments could therefore be attributed to the inducing action of the mesoderm.

SERIES 2. EXPLANTS OF THE DORSAL LIP FROM THE EARLY GASTRULA INTO YOUNG GASTRULA ECTODERM

In a series of explants of which three survived, the dorsal lip was excised from the early gastrula (Harrison's stage 10), placed in alkaline saline until its cells had disaggregated, then returned to normal saline to reaggregate. It was wrapped in early gastrula ectoderm and cultured for 10–12 days.

Eight control explants were made in addition, using normal, intact dorsal lip.

Results

The different types of inductions obtained in experiments and controls are summarized in Table 1. When actual numbers, not percentages, were analysed statistically, it was found that there was no significant difference between experiments and controls.

Table 1. *Neural inductions and mesoderm differentiation in explants of dorsal lip from the early gastrula*

1	2		3		4		5		6		7		8		9	
	Total explants		Total inductions		No. inductions, brain		No. inductions, eye		No. inductions, neural tube		No. inductions, neuroid		Mesoderm, notochord		Mesoderm, somites	
		%		%		%		%		%		%		%		%
Experiments	13	100	10	76·9	5	50·0	1	100·0	9	90·0	3	30·0	7	33·5	4	30·8
Controls	8	100	6	75·0	5	83·3	1	16·67	6	100·0	2	33·3	6	75·0	3	36·7

Note. In this and following tables, total inductions are expressed as percentage of explants, and other inductions as percentage of total inductions.

Certain failures of bilateral symmetry were noticed in the nervous tissue of both experimental and control explants. The neural tube, for instance, frequently had a very small, circular lumen, and whichever wall lay next to partly or completely differentiated somite tissue was thickened. Pl. 2, fig. A shows an interesting case where the neural tube lies on its side with respect to the epidermis, having formed a thickened 'lateral' wall adjacent to the somite tissue lying between it and the notochord. This is in accordance with Holtfreter's observation (1933*a*) that the symmetry of the neural tube is determined by the position of somites and notochord.

The brains formed were frequently asymmetrical or radially symmetrical, and eye inductions were nearly always single. There was only one explant containing paired eyes, and these were contiguous in the mid-line owing to the absence of an infundibulum.

The absence of any significant difference between the results for experiments and controls in Table 1 supports Holtfreter's assertion (1944*b*) that disaggregation of the cells of the organizer does not affect its ability to induce a regionally differentiated nervous system. Although too much stress should not be laid on statistical differences where such small numbers are concerned, it is at least clear that recognizable brain, sense organs and neural tube can be induced by the reaggregated organizer. The degree of differentiation was not as complete as Holtfreter describes in his implants, nor had the tissues been organized into anything like a complete neural axis, but such differences are usual when explants and implants are compared (cf. Gallera, 1948; Pasteels, 1949). The essential result here is the similarity between inductions by disaggregated and by normal organizer.

SERIES 3. EXPLANTS OF THE COMPLETE ARCHENTERON ROOF FROM THE LATE GASTRULA

For these experiments, embryos of Harrison's stages 12 and 12½ were used (small yolk-plug or slit blastopore stages). After deflecting the dorsal ectoderm, the whole archenteron roof mesoderm was removed in one piece for explantation.

(*a*) 1949 *series*

Owing to shortage of material, ventral ectoderm from the same late gastrula was used to enwrap its explanted archenteron roof: this made it possible to use only one embryo per explant instead of two.

In some 100 experiments, of which seventy-three survived to give clear results, the archenteron roof tissue was disaggregated and reaggregated as described previously, then explanted into the ventral ectoderm and cultured for 14 days.

Results (*Table* 2)

Fifteen (i.e. 40·55 %) of the controls elongated to resemble an embryonic axis. In two of these a normal-looking head developed, including a well-differentiated eye with lens and iris. There was also a tail outgrowth with a fin. The other explants were either spherical or irregular in shape.

In the experimental series only eleven (i.e. 30·55 %) formed anything resembling an embryonic axis, and none of these was as complete as the two controls mentioned above. Some had distorted tail outgrowths, and in others gills or balancer were recognizable.

The morphological features of the nervous tissues induced were not noticeably different as between experiments and controls. In both series they tended to be incomplete and asymmetrical. The brain was usually radially symmetrical or asymmetrical with one thickened wall next to a sense organ (eye or otic capsule). Pl. 2, fig. B, shows a very small piece of brain tissue thickened on the side next to the single eye which it has induced. Pl. 2, fig. C, shows a piece of hindbrain, also thickened on one side, next to the single otic capsule. Both of these cases were explants of disaggregated organizer, but there were similar cases among the controls.

The sense organs were nearly always single: in only one experimental explant, and one control, were there paired eyes.

The neural tubes in these explants were also atypical. There was not a single case where an elongated, bilaterally symmetrical tube had been formed. Instead, short thin-walled tubules or solid rods of neuroid cells were the rule.

Since regional differentiation implies a distinction between head and trunk nervous system within the same unit of tissue, a comparison was made between the numbers of experiments and controls which had both brain and neural tube in the same explant (col. 9 of Table 2). These figures were found not to differ significantly.

The numbers of brain, eye and neural tube inductions obtained are given in Table 2. When analysed statistically they show no significant difference between experiments and controls. But the total number of inductions of all kinds was significantly lower in the experiments. This may have been due in some cases to excessive treatment with alkali, since in several explants where there had been no induction the mesoderm appeared dead. It is interesting that dead mesoderm never appeared to induce: on the contrary, induction was invariably associated with the survival and differentiation of the mesoderm into notochord or mesenchyme.

Table 2. *Neural inductions in explants of complete archenteron roof*

1	2		3		4		5		6		7		8		9	
	Total explants		Total inductions		No. inductions, brain		No. inductions, eye		No. inductions, neural tube		No. inductions, neuroid		Embryonic axes		Both brain and neural tube	
		%		%		%		%		%		%		%		%
Exps.	73	100	36	49·3	13	36·11	5	13·89	17	47·22	5	13·89	11	30·55	7	19·44
Controls	48	100	37	77·1	14	37·8	7	18·92	10	27·03	13	35·15	15	40·55	12	32·44

The above results show quite clearly that the qualitative inducing powers of the archenteron roof of the late gastrula are not impaired by disaggregation of its cells. All the recognizably different types of neural tissue arose both in the experiments and in the controls. Brain, sense organs, and neural tube could be distinguished in both series, although they tended to be incompletely differentiated and irregular in shape. The possible reasons for this irregularity, typical of all induction in explants, are discussed later.

In both experiments and controls, the frequencies of brain and eye inductions were surprisingly low. This was probably due to the advanced age of the ectoderm, since Holtfreter (1938) found that the ventral ectoderm of the late gastrula has lost some of its competence for neural differentiation, and Waddington (1936) showed that at this stage even the lateral ectoderm is not as reactive to the organizer as is the dorsal ectoderm. So in 1950, when material was more abundant, a few additional explants were made using ectoderm from an early gastrula to enclose the archenteron roof.

(*b*) 1950 *series*

Ten explants were made of untreated archenteron roof into early gastrula ectoderm. Only four survived, but all of these had formed brain, and three had eyes also. In all three cases the eyes were paired, but contiguous in the mid-ventral line owing to the absence of the infundibulum.

Two explants showed complete, symmetrical embryonic axes: in fact, they differed from normal embryos only in the absence of endoderm.

Discussion

The markedly higher percentage of brain and eye inductions in this series than in the 1949 series indicates that early gastrula ectoderm has a much greater competence for neural differentiation than late gastrula ventral ectoderm. The cause of this lowered competence in the late gastrula ectoderm has never been explained: but it could possibly be due to its lower potentiality for growth than young gastrula ectoderm. The piece excised from the late gastrula was what would normally form only the ventral epidermis, whereas that used from an early gastrula represented the whole of the presumptive epidermis, as well as presumptive neural plate. These areas would be expected to show more growth under the influence of an organizer than would ventral epidermis alone.

The ability of the series (*b*) explants to form symmetrical embryonic axes is also worthy of comment. It indicates that individuation depends in part on factors residing in the dorsal ectoderm.

SERIES 4. EXPLANTS OF SEPARATE THIRDS OF THE ARCHENTERON ROOF FROM THE LATE GASTRULA

In the following series, control explants were carried out to test for regional differences in the normal archenteron roof, before investigating the effect of disaggregation on the organizer at this stage.

(*a*) *Control explants of separate thirds of the archenteron roof*

The archenteron roof of the late gastrula (stage H. 12–12½) was divided into equal sized anterior, middle and posterior parts. Each part was explanted into one half of the ectodermal area from the early gastrula.

Results

After 10–12 days little differentiation was visible externally, and only very few explants showed any elongation or tail outgrowths (see Table 3, col. 8). The degree of external differentiation did not depend on the region of the archenteron roof explanted. In section, brain tissue was identified in 47·9 % of the inductions, eye tissue in 35·4 % and neural tube in 31·6 %, averaging the figures for the three types of explant (Table 3). When the frequencies of these types of induction were compared as between different archenteron roof thirds, no significant difference was shown. The posterior thirds appeared just as capable of inducing midbrain structures as the anterior two-thirds, and conversely, the anterior third could induce neural tube.

Pl. 2, fig. D, shows a symmetrical brain, with an eye cup, induced by the posterior third of the archenteron roof.

In all these explants there was the same asymmetry and incomplete differentiation as had been seen in series 2 and 3. Pl. 2, fig. D, shows the only case where the brain was bilaterally symmetrical. Further, the total number of inductions was very low. It was thought that this might be due to some factor of size, limiting the inducing powers of small organizer pieces. So a second series of control explants was made, in each of which three anterior, three middle, or three posterior thirds were combined, so that the total mass of mesoderm was equal to a complete archenteron roof.

Table 3. *Neural inductions by separate thirds of archenteron roof* (*group a*)

1	2		3		4		5		6		7		8	
Region	Total explants		Total inductions		No. inductions, brain		No. inductions, eye		No. inductions, neural tube		No. inductions, neuroid		No. with tail outgrowth	
		%		%		%		%		%		%		%
Ant. third	30	100	8	26·7	5	62·5	3	37·5	1	12·5	1	12·5	2	25·0
Mid. third	39	100	16	41·0	7	43·75	8	50·0	3	18·75	0	0	8	50·0
Post third	29	100	16	55·2	6	37·5	3	18·75	10	62·5	0	0	3	18·75
Av.	32·7	100	13·3	40·9	6	47·9	4·6	35·4	4·6	31·6	0·3	4·2	4·3	31·25

(*b*) *Control explants of groups of three similar thirds of the archenteron roof*

In this series, the whole ectodermal area from an early gastrula was used to wrap each group of three archenteron roof thirds.

Results

The total percentage of inductions (60%; see Table 4) was much higher than in series (*a*). Moreover, some regional differences were now evident between the different organizer thirds, since anterior thirds gave a significantly higher percentage of brain and eye inductions than middle or posterior thirds. This falls into line with ter Horst's (1948) and Mangold's (1929*a*) results obtained with the archenteron roof of the early neurula.

Having found conditions in which regional specificity could be demonstrated in different parts of the normal organizer, it was possible to test the effects of disaggregating its cells.

(*c*) *Explants of groups of three similar archenteron roof thirds after disaggregation in alkali*

Anterior, middle and posterior thirds of the archenteron roof were disaggregated separately in alkaline saline. The cells from three anterior, three middle, or three posterior thirds were then combined and explanted into the complete ectodermal area from an early gastrula.

Results

Fewer of these explants showed elongation or tail outgrowths after 10–12 days' culture than had either of the control groups. On histological examination, however, it was found that the total percentage of inductions (73·2%; see Table 5) compared favourably with group (*b*). The frequencies of brain, eye and neural tube inductions by corresponding thirds in groups (*b*) and (*c*) did not differ significantly (cf. Table 4 with Table 5).

Table 4. *Neural inductions by groups of three archenteron roof thirds from same region* (*group b*)

1	2		3		4		5		6		7		8	
Region	Total explants		Total inductions		No. inductions, brain		No. inductions, eye		No. inductions, neural tube		No. inductions, neuroid		No. with tail outgrowth	
		%		%		%		%		%		%		%
3 ant. thirds	13	100	3	23·1	2	66·7	0	0	1	33·3	0	0	2	66·7
3 mid. thirds	13	100	9	69·2	9	100·0	8	88·89	1	11·1	0	0	4	44·4
3 post. thirds	9	100	9	100·0	3	33·3	1	11·1	6	66·7	0	0	2	22·2

Table 5. *Neural inductions by group of three* (*reaggregated*) *archenteron roof thirds from same region* (*group c*)

1	2		3		4		5		6		7		8	
Region	Total explants		Total inductions		No. inductions, brain		No. inductions, eye		No. inductions, neural tube		No. inductions, neuroid		No. with tail outgrowth	
		%		%		%		%		%		%		%
3 ant. thirds	23	100	15	65·2	9	60·0	4	26·7	8	53·5	12	80·0	7	46·7
3 mid. thirds	26	100	18	69·2	10	55·5	5	27·8	8	41·1	15	83·3	5	27·8
3 post thirds	22	100	19	86·4	7	36·8	1	5·3	9	47·3	12	63·2	3	15·8

Observations were also made on the histological differentiation of the mesoderm, and it was noticed as in series 3 (*a*), that whenever a neural tube had been induced some notochord tissue had formed. Muscle tissue also formed occasionally, but was never organized into a regular series of somites. However, the ability of trunk mesoderm to form notochord showed for certain that it had not been killed by the alkali treatment, and also indicated that it had undergone considerable reorganization after reaggregation.

An analysis of the data of Table 5 showed that there was no significant difference between anterior, middle and posterior thirds as regards the numbers of each type of induction given (cols. 4–7). So judged on a quantitative basis, it appears that the regional specificity of the different parts of the organizer had been destroyed by disaggregation. It must, however, be emphasized that there is no sharp distinction between the results of group (*b*) and group (*c*): untreated posterior thirds induced anterior nervous tissue just as often as disaggregated posterior thirds, but because middle and anterior thirds gave more brain inductions in series (*b*) than after disaggregation in (*c*), differences between the three regions reached the level of significance in (*b*). The alkali treatment reduced the incidence of cephalic inductions by anterior parts of the organizer, and this was responsible for the apparent loss of regional specificity in the separate thirds in (*c*) when the types of inductions which they gave were compared.

Since all types of neural tissue in these explants tended to be irregular in shape, they were identified by their histological character rather than their form. For instance, a neural vesicle was classed as brain not merely because its diameter was larger than a neural tube, but because of having extremely thickened walls consisting of several layers of cells with small, closely packed nuclei orientated towards the lumen of the vesicle. This is in contrast to a normal neural tube, which has few layers of cells, the innermost layer containing large, elongated nuclei.

The nerve tubes in these explants were atypical: the lumen was circular instead of the normal elongated dumbbell shape, and the cells were orientated in radial instead of bilateral symmetry. The whole tube seldom extended for more than ten sections (100 μ). There was one case where a solid neural rod had formed in the centre of the explant, evidently through differentiation of the mesoderm, not the ectoderm.

An attempt was made to compare the degree of differentiation of the neural tissue induced by different organizer thirds. This was not easy, since some explants had been cultured for longer than others which had been fixed early owing to signs of ill-health. However, inductions by the middle third of the organizer were, on the whole, the most complete and easily recognizable: in contrast, inductions by the anterior third appeared the least successful, and were sometimes rather doubtfully neural. It is interesting that Okada & Hama (1945) found that the anterior one-third of the late gastrula archenteron roof did not induce at all when implanted into the neurula. They did not, however, try explanting it.

Summary of results of series 4

The results in group (*b*) demonstrate the existence of a regional specificity in different thirds of the archenteron roof at the late gastrula stage. This specificity showed itself by quantitative differences in the frequency of brain, eye and neural tube induced by the separate thirds. Although no qualitative regional differences could be demonstrated—the posterior third was able to induce the same range of structures as more anterior parts—the quantitative differences were large enough for one legitimately to distinguish three regions in the organizer at this stage. The conclusions of Okada & Hama (1945), referred to on p. 20, are, therefore, confirmed.

Comparing the quantitative results with those of ter Horst (1948), it appears that in the present experiments a more posterior region of the archenteron roof was able to induce an eye. In her results, no eye was induced by the two posterior fifths of the archenteron roof. However, at stage H. 12, these parts would not yet have invaginated (cf. Pasteels, 1943). Mesoderm which was in the posterior third of the archenteron roof in the late gastrula would have reached the middle fifth of it by the early neurula stage. This region, ter Horst found, induced eye in 33% cases: exactly the same figure was obtained in group (*b*) here for the middle thirds of the archenteron roof.

Comparison of the results in group (*b*) and (*c*) shows that after disaggregation the power of the archenteron roof to induce brain or eye tissue is reduced in the anterior two-thirds, and that therefore, regional differences, detected by the present criteria, are no longer apparent. But it is not justifiable to conclude that regional specificity has been entirely destroyed: some more accurate means of measuring it is desirable.

It was also observed that inductions were more successful in control group (*b*) than in group (*a*), and that regional differences became apparent in (*b*) although they could not be detected in (*a*). The possible reasons for this result are discussed later.

SERIES 5. IMPLANTATION OF DISAGGREGATED DORSAL LIP INTO THE EARLY GASTRULA

The following experiments were carried out to compare the degree of differentiation and bilateral symmetry attained by induced neural tissues in implants with that attained in explants where, as has been seen, the formations were far from normal.

The dorsal lip was excised from an early gastrula (stage H. 10) and disaggregated by the usual alkali treatment. The reaggregated cells were implanted under the prospective ventral ectoderm of an early gastrula (Mangold's 'Einsteckung' technique). It was found best to insert the tissue through an incision made in the dorsal ectoderm near the anterior end of the embryo. The implant then usually remained in its anterior position, partly through becoming fused to the ectoderm as the wound healed, and partly because the embryo had flattened dorso-ventrally after the removal of its vitelline membrane.

Holtfreter (1944*b*) used the material from several blastopore lips for his implants, but in the present experiments the material from one lip was found to be sufficient, since very few cells were lost during the alkali treatment. More than one reaggregated dorsal lip formed, in fact, too large a piece of tissue to implant conveniently.

Five control implants were also made, using untreated blastopore lip tissue.

Results

In fourteen out of fifteen experiments, and in all the controls, inductions were obtained. One control formed a complete secondary larva ventrally, but there was no comparable case among the experiments, though many of them formed fairly definite secondary axes. In six of the experimental series there was secondary hindbrain, but none had formed forebrain or midbrain. Even in the controls, the one complete larva was the only secondary induction which included anterior parts of the brain. This is in striking contrast to Holtfreter's claims to have obtained complete heads induced by disaggregated dorsal lip.

Tables 6 and 7 give in detail the tissues present in the secondary axes of both experiments and controls. It is not possible to make statistical comparisons on such few data, but one gains the general impression that, apart from the one complete

Table 6. *Neural inductions, and mesoderm differentiation, in control dorsal lip implants into early gastrulae*

Exp.	External appearance of induced axis	Secondary inductions							Mesoderm			
		Fore-brain	Mid-brain	Hind-brain	Eye	Otic	Neural tube	Neuroid	Noto-chord	Somites	Kidney	2° gut
1	Anterior tail outgrowth	−	−	−	−	−	+	−	+	+	−	+
2	Anterior tail outgrowth	−	−	+	−	−	+	+	+	+	−	−
3	Complete secondary larva	+	+	+	+	+	+	−	+	+	+	+
4	Anterior tail outgrowth: reversed	−	−	+	−	+	+	−	+	+	−	+*
5	Ventral axis	−	−	−	−	−	+	+	+	+	−	−

* Continuous with gut of host.

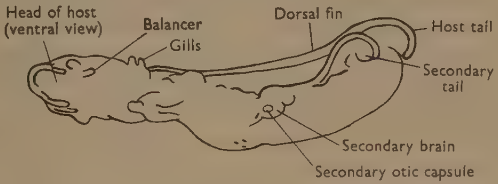
Table 7. *Neural inductions, and mesoderm differentiation, in reaggregated dorsal lip implants into early gastrulae.*

Exp.	External appearance and position of induced axis	Secondary inductions							Mesoderm			
		Fore-brain	Mid-brain	Hind-brain	Eye	Otic	Neural tube	Neuroid	Noto-chord	Somites	Kidney	2° gut
1	Dorsolateral, fused with host's	−	−	+	−	−	+	+	+	+	−	+
2	Well-defined, lateral	−	−	+	−	+	+	+	+	+	−	+
3	Accessory neural folds	−	−	−	−	−	+	+	+	+	−	+
4	Accessory neural folds and tail	−	−	−	−	−	+	−	+	+	−	+
5	Ventral tail outgrowth	−	−	−	−	−	+	−	+	+	−	−
6	Lateral axis	−	−	−	−	−	+	−	+	+	−	+
7	Ventral axis (short)	−	−	−	−	−	+	+	−	+	−	−
8	Ventral axis (nearly full length)	−	−	+	−	−	+	+	+	−	−	−
9	Dorsolateral, posterior	−	−	+	−	+	+	−	+	+	−	+
10	Ventral, posterior	−	−	−	−	−	+	+	+	+	−	−
11	Dorsal tail outgrowth	−	−	−	−	−	+	−	+	+	−	−
12	Lateral, posterior	−	−	−	−	−	+	+	+	+	+	+*
13	Not evident	−	+	+	−	−	+	−	+	+	−	−
14	Lateral, well-defined	−	−	+	−	−	+	−	+	+	+	+
	Total	0	1	6	0	2	14	7	13	13	2	8

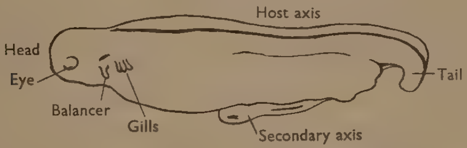
* Fused to gut of host.

larva, the controls did not feature any more complete or successful inductions than the experiments. Hindbrain was induced in two further controls, and in six out of the fourteen experiments. Other neural tissue consisted only of neural tube, while the mesoderm itself formed notochord and somites.

The nervous tissue in all these secondary inductions was elongated antero-posteriorly and was bilaterally symmetrical. Histologically it was much more regular than any inductions obtained in explants. Although both neural tube and brain consisted usually of fewer cells than had been observed in explants—because the embryos had been cultured for a shorter time (6–9 days) and cell-multiplication had not, therefore, proceeded so far—these cells were arranged compactly, in a definite orientation with respect to the lumen.



Text-fig. 1. Lateral secondary axis induced by reaggregated organizer tissue.



Text-fig. 2. Ventral secondary axis induced by reaggregated organizer tissue.

The position of the secondary axes varied in both experiments and controls. Though the implant had always been placed ventrally, it had evidently been carried dorsalwards in some cases. This phenomenon has been noticed in implants, by Yamada (1937) and by Holtfreter (1944*b*), who postulated that there was some attractive force from the host's axis which drew the graft towards it. Recently, Dalcq & Minganti (1949) have observed the migration of grafted cells towards the host's axial mesoderm. However, it is more probable that in the present experiments the graft was shifted by the invagination movements of the host.

In the experimental series it was noticeable that dorsally placed secondary axes were better differentiated than ventral ones. For instance, the secondary axis of Text-fig. 1 shows the outline of a brain, and also a dorsal fin. The ventral axis in Text-fig. 2 is by comparison far less definite. The data of Table 7 show further that brain was more frequently induced in lateral than in ventral secondary axes.

The mesoderm of the lateral axes also had a more symmetrical organization than in ventral axes. There were paired somites in all of them, whereas if somites were present in the ventral axes they formed a single block in the mid-line. A further observation was that a secondary archenteron was associated with all but one of the lateral inductions, though none ever formed with the ventral inductions. This suggested that the differentiation of the host endoderm might influence the layout and symmetry of the grafted mesoderm.

In order to investigate further the possible action of the endoderm on the graft, some implants were made into ventral halves of early gastrulae. These are described in the next section.

SERIES 6. IMPLANTS OF DISAGGREGATED DORSAL LIP TISSUES INTO VENTRAL HALVES OF EARLY GASTRULAE

The dorsal lip and complete mesodermal area were removed from an early gastrula, and disaggregated in alkaline saline. The remaining part of the embryo, to be used as host, was left in the operating dish resting in such a position that its own weight pressed the cut surfaces together. After 2–3 hr., the wound had partially healed, leaving a small area through which the reaggregated dorsal lip tissue could be inserted into the blastocoel. However, subsequent healing was seldom successful: only two operations succeeded out of eleven performed.

Seven ventral halves of early gastrulae were cultured without grafts, as controls.

Results

The two implants which survived gave rise to elongated embryonic axes on the dorsal side of the host. Pl. 2, fig. E, shows the appearance of one of them after 7 days. On histological examination, the neural axis of this embryo was found to consist only of nerve tube with some neuroid tissue at the anterior end. In the second embryo, however, there was midbrain, hindbrain and otic tissue. The otic region is shown in Pl. 2, fig. F. Both embryos possessed symmetrically arranged paired somites on either side of a notochord. The endoderm had differentiated into a normal gut.

The control half-embryos were all spherical in shape after 7 days' culture. The dorsal ectoderm had expanded by growth, but no neural tissue had formed.

The method of operation used in these experiments was unsatisfactory because so few embryos healed successfully. Filatov (1937) experienced the same difficulty when implanting eye cups into ventral halves of early gastrulae. However, it is desirable to carry out more experiments of this type, since the ventral half of a gastrula is an ideal host, offering normal mechanical conditions for the invagination of grafted mesoderm but having no axis of its own to exert any inducing action.

The two positive results were interesting because they showed that paired somites will form in the presence of endoderm. This is in contrast to the irregular single mass of somite tissue which was the most that was obtained in explants even of normal organizer when no endoderm was present.

The conclusion already suggested by the observations in series 5 is, therefore, confirmed: namely, that the endoderm exerts some influence on the individuation of the somite mesoderm in amphibian embryos.

DISCUSSION

The most clear-cut result of the present work has been the confirmation of Holtfreter's observations (1944*b*). It has been shown that in both explants and implants the dorsal lip can both differentiate and induce, exhibiting regional properties, even after its cells have been disaggregated in alkali, and reaggregated in random order. Evidently its antero-posterior specificity can be altered according to the new arrangement of the cells. Rather more surprising, however, was the observation in series 3 that even at as late a stage as the end of gastrulation, rearrangement of the archenteron roof cells in this manner did not destroy the regional properties of the tissue as a whole. At this stage Okada & Hama (1945) have been able to demonstrate differences in inducing power in different parts of the archenteron roof explanted separately, while our own data in series 4 show differences in the percentages of brain and eye inductions by explants of mesoderm derived from three different regions of the archenteron roof. To explain the results of series 3, then, it is necessary to assume that these regional differences are reversible, and can be re-established after they have been experimentally disturbed.

However, the evaluation of qualitative differences by means of quantitative data must always be a somewhat doubtful procedure, especially with operative work on amphibian embryos, where it is seldom possible in one season to collect enough data for a convincing statistical analysis. Some of the morphological features of the present results have also suggested that the quantitative data should be accepted with reserve.

To begin with, it should again be remarked that all the nervous tissue induced in explants was atypical, so that the absence of differences between experiments and controls simply meant that inductions after disaggregation were no less typical than before disaggregation: it did not imply that they were bilaterally symmetrical in either case. It was, therefore, a negative observation: a failure to detect regional alterations, and not a proof that none had taken place. Secondly, it should be noted that some inductions which were not definitely recognizable as either brain or neural tube had to be classed as 'neuroid', and were not included in the evaluation of the regional properties of the organizer. This will have tended to produce uniformity in results which might otherwise have shown differences between experiments and controls.

The abnormality of inductions obtained in explants has been remarked on by Gallera (1948) and Pasteels (1949). Chuang (1939), though he claims to have obtained one or two symmetrical brain inductions by non-specific inductors, depicts cases which show quite clearly the lack of symmetry in explants as compared with implants. None of these authors has attempted to explain this phenomenon.

Reasons might, however, be suggested for some of the abnormalities which arose in the present work. In the asymmetrical neural tubes, for example, the thickening of the wall on the side adjacent to somite mesoderm, in accordance with Holtfreter's observations (1933*a*), could be explained by his view that the somites have some influence on the differentiation of the neural tube. The occasional radial symmetry of brain tissue might be due to the fact that a complete sphere of ectoderm had come under the influence of the organizer, whereas in a whole embryo only the dorsal ectoderm can be affected, since the ventral ectoderm is separated from the organizer by endoderm. In addition, it could be suggested that the development of either radial or bilateral symmetry is controlled by mechanical factors. In a whole embryo, where the presumptive neural plate is stretched as a roof over the yolky ventral tissues, the orientation of the neural folds must to some extent be governed by the direction in which overall elongation is occurring in the embryo. Clearly it would be a mechanical feat of some difficulty for transversely orientated neural folds to grow up and then close across the ever-increasing longitudinal axis of the embryo to meet in the dorsal mid-line. The normal mode of neurulation is much easier to accomplish. In an explant, on the other hand, there are no mechanical restrictions due to ventral tissues, so that elongation could occur in any direction: the fact that it may occur equally in all directions and result in radial symmetry is not surprising.

In contrast to the brain and neural tube, no asymmetries were observed in individual sense organs. This is probably because their intrinsic symmetry is normally radial, not bilateral, and so is not upset by the conditions of explantation. It should also be noted that the differentiation of sense organs depends chiefly on histological changes: foldings of tissue and other morphogenetic movements are on a very small scale.

One abnormal feature of the sense organs, however, was that they were seldom paired in explants. It was noticed particularly that the eyes were nearly always single. Adelmann (1936), in a review of the causes of cyclopia, has pointed out that the eye cups invariably fuse in the mid-ventral line if for any reason the infundibulum fails to develop. Mangold (1936) and Nieuwkoop (1946) reported instances where absence of foregut endoderm was associated with fusion of the eye cups and failure of the infundibulum to develop. The absence of paired eyes in the present explants might, therefore, be explained by the fact that they contained no endoderm.

It was noticed that the mesoderm was itself able to reorganize to a considerable extent after reaggregation. In the explants of series 3 and 4, as has already been remarked (pp. 24 and 27), some notochord tissue was invariably present when neural tube had been induced, whereas brain inductions were accompanied only by mesenchyme or by undifferentiated mesoderm. It appears, then, that even at the end of gastrulation the differentiating properties of the mesoderm cells can be modified, so that they can form an axial notochord even after having been disarranged by alkali treatment. It would seem also that notochord-formation is essential to the induction of neural tube. This gives some support to Yamada's hypothesis (1950) that the induction of trunk nervous system requires special mechanical conditions, one of which is the elongation of the mesoderm which occurs when it forms a notochord.

Another observation by Yamada (1937) was that somites formed preferentially in explants which included some notochordal tissue, and differentiated more regularly under the influence of the notochord. In the present explants, on the other hand, muscle tissue only rarely formed (cf. Okada & Hama's findings, 1945), and it was never organized into a series of somites, even though notochord was often present. Normal somites were, however, formed by implanted organizer tissue, and their pairing was dependent on the presence of the notochord.

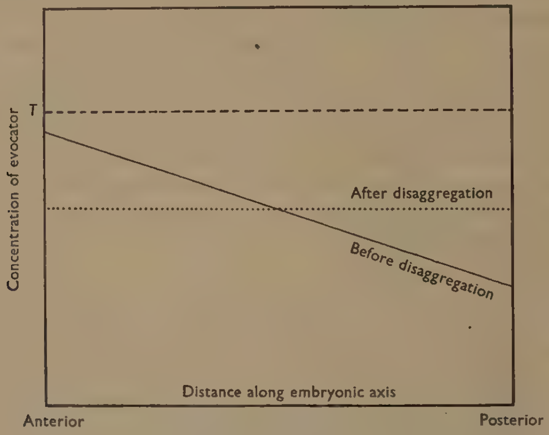
The experiments of series 5 and 6 confirmed the observations of many other workers that the conditions of implantation control the development of a normal symmetrical embryonic axis. But the superior differentiation of lateral secondary axes as compared with ventral ones was contrary to Lopaschov's findings (1941). He obtained the best inductions by ventral implants. However, since his grafts were made into late neurulae, they were unaffected by gastrulation and neurulation movements of the host tissues. In the present experiments, on the other hand, where the host was an early gastrula, a graft which came to lie laterally would be assisted in spreading forwards by the similar movements taking place in the host's mesoderm. A ventral implant would, however, tend to be carried towards the posterior by the rotation of the invaginating endoderm (Nicholas, 1945), and the cells of the gut floor would then pile up on top of it. It is clear that under these conditions the implant could not spread forwards or induce as successfully as a lateral implant, so that the present results are readily explained. Mangold (1929*b*) and Spemann (1931*b*) also observed a correlation between the success of inductions and the degree to which the graft had been able to carry out normal morphogenetic movements unimpeded by host tissues.

There are indications that in contrast to the impeding action of ventral ectoderm, dorsal endoderm can assist the individuation of a graft. For in series 5, where the host dorsal lip had been removed, two successfully implanted organizer reaggregates differentiated much better than any of the explants, forming paired somites as well as a notochord. It had also been noticed in series 5, that all the well-differentiated secondary axes were associated with a secondary archenteron. It is unknown whether the endoderm exerts any control over the differentiation of other tissues in amphibia. Although observations have been made on embryos lacking endoderm by Mangold (1936) and Nieuwkoop (1946), and on endoderm isolates *in vitro* by Stableford (1947) and Nicholas (1945), none of these studies has given any evidence that the endoderm acts as an inductor in the physiological sense. It remains possible, however, that it plays a purely mechanical role, since its active forward and lateral immigration would assist the spreading of the dorsal mesoderm in contact with it.

The morphological observations in the present work, then, show a striking ability of the disaggregated mesoderm to reorganize itself, to differentiate and to induce various types of nervous tissue. They also point to the importance of the endoderm in amphibian development. This tissue, which occupies more than half the volume of the early amphibian embryo, has received scant attention in the literature. It is evidently concerned in the formation of the infundibulum, as well as possibly controlling the individuation of the mesoderm.

To return to the quantitative data obtained in this work: it was remarked in the Introduction that if Holtfreter's observations on the inducing powers of the reaggregated organizer were confirmed, it would be difficult to maintain any theory of regional differentiation which depended upon a property of the organizer tissue as a whole. However, a closer examination of the data from the explants shows that they are explicable on a simple 'gradient' theory.

In series 4, the appearance of significantly different numbers of cephalic and trunk inductions in explants of different thirds of the archenteron roof has been taken as evidence for regional specificity in the inductor. On this criterion, untreated thirds of the archenteron roof explanted singly showed no regional specificity but when three similar thirds of the archenteron roof were combined in each explant, the controls showed regional specificity while the experimental (disaggregated) series did not.



Text-fig. 3. Hypothetical linear concentration gradient of a single evocator within the archenteron roof.

Text-fig. 3 depicts the simplest imaginable gradient system within the archenteron roof. A single evocator is assumed to be responsible for all types of neural induction, and its concentration is shown as a linear gradient with the highest point anterior. The concentration T represents the threshold for induction of brain: normally reached only in the anterior parts of the organizer. Though one could assume that a number of thresholds exists, each responsible for a certain level of neural induction along the anterior-posterior axis, it is only the brain level which is morphologically distinct and, therefore, of interest to us. Now since at the late gastrula stage regional differentiation in the mesoderm is still labile, and anterior pieces may in certain circumstances induce trunk nervous system (Spemann, 1931*b*; Hall, 1937; etc.), it is necessary to assume that the threshold T has not yet been reached at this stage. Alternatively, one would have to suggest that transplanted tissue sometimes suffers a loss of evocator so that the concentration falls below T. This would be comparable to the 'lowering of morphogenetic potential' invoked by Dalcq (1940) to explain the subnormal differentiation of some organizer transplants.

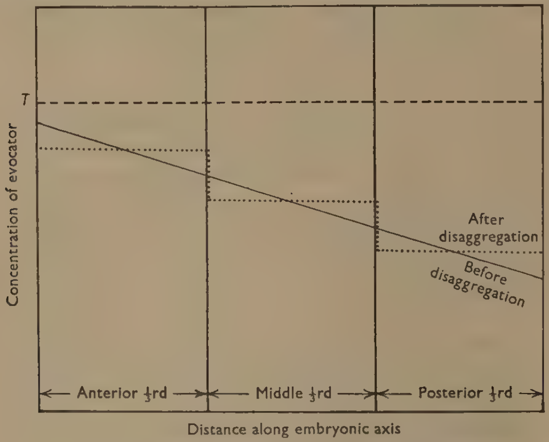
To account for the absence of regional specificity in the series 4 (*a*) explants (single thirds), it could be suggested that the anterior third of the organizer normally depends upon the other two-thirds for supplies of essential substrates. We have just seen that the lability of anterior mesoderm requires the assumption that the evocator concentration for one reason or another lies below T immediately after it has been isolated. It is possible that the isolated anterior third is sometimes unable to synthesize sufficient evocator to induce a brain, while an isolated posterior third may, on the other hand, be capable of synthesizing more than its normal concentration of evocator because it is no longer giving up substrates to anterior mesoderm. This will result in brain inductions by some posterior thirds (cf. Table 4). These two effects together may have been responsible for reducing the observed regional differences between the two extremities of the archenteron roof below the level of statistical significance, in series 4 (*a*).

The appearance of regional specificity when three archenteron roof thirds are combined, as in series 4 (*b*), is explicable if two more anterior thirds provide either additional evocator or additional substrates to the original anterior piece, enabling the threshold concentration T to be reached. The final evocator concentration in middle and posterior thirds must also be higher when three pieces are combined than in single pieces, but concentration differences between these and the anterior explants could still remain large enough for significantly more of the latter to give brain inductions. It is interesting to note that if any three numerical values are assigned to the evocator concentrations of the three organizer regions, and these are added when three similar thirds are combined, arithmetical differences are bound to be greater between groups of three thirds than between single thirds, when different regions are compared. So theoretically, a very simple numerical explanation could be given for the appearance of regional specificity in series 4 (*b*) when it was not apparent in (*a*).

It has been noticed by other workers that the nature of neural inductions depends on the size of the inducing tissue. Lopaschov (1941) obtained variation in the type of neural tissue induced, by explanting different numbers of dorsal lips. Dalcq & Lallier (1948) report that large implants give more complete inductions than small ones. On the other hand, Marx (1925) was unable to find any differences in the inductions by large and by small implants: it is clear, then, that further work needs to be carried out before a decision can be reached as to the significance of organizer size in regional determination.

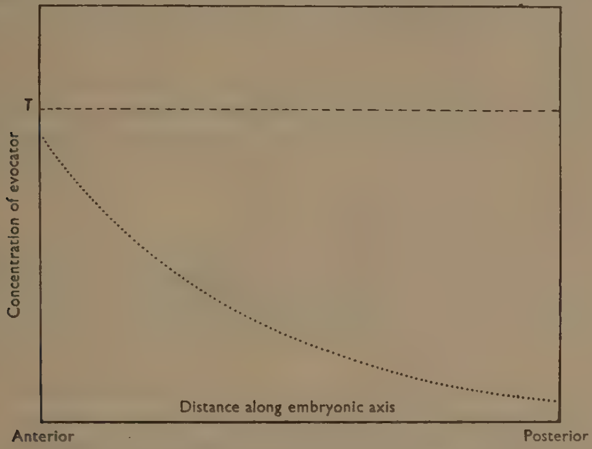
Disaggregation of the cells of the archenteron roof, followed by their reaggregation in random order, would level out the gradient of Fig. 3. In series 2, 3 and 5, where all the cells were recombined as one piece, the resultant evocator concentration could be represented as a horizontal line at the average value of the original gradient. However, since according to the data the regional properties of the tissue appear unchanged, it is necessary to postulate that the gradient is re-established after explantation. The substrates required for this process should be available, since all the material from the original archenteron roof is present. What determines the anterior end of the reaggregated tissue, however, remains unexplained.

A different situation will arise when separate thirds of the archenteron roof are disaggregated, for if the concentration gradient is levelled out to an average value in each region, there will be much sharper differences in evocator concentration between adjacent boundaries of the different regions (Text-fig. 4) than there were before disaggregation. At first sight, then, it seems that regional differences should be increased, and not decreased as in series 4 (*c*). However, the recognition of these differences depends upon the number of explants in which the threshold concentration (T) for brain induction is reached. It was observed previously that disaggregated anterior and middle thirds gave much fewer cephalic inductions than controls: this suggests that in many of these explants the average concentration of evocator after flattening of the gradient lay well below T. This would mean that the original concentration gradient had been steep. No such reduction of cephalic inductions occurred in disaggregated posterior thirds, however, suggesting that the gradient in this region had not been very steep. So the curve of Text-fig. 5 probably gives a truer picture of the type of gradient which may be responsible for regional differences. It is consistent with Waddington & Yao's hypothesis (1950) that liberation of evocator begins in the presumptive anterior mesoderm of the early gastrula and continues throughout gastrulation. The concentration would in this case always be higher in the anterior mesoderm, where it would also increase proportionately more quickly: a process which would result in the logarithmic curve of Text-fig. 5.



Text-fig. 4. Effect of disaggregating separate thirds of archenteron roof on hypothetical evocator concentration gradient.

It can be concluded, then, that all the results obtained in the present work are explicable on the assumption that regional differentiation is controlled by a single gradient in the organizer tissue. It is, however, very unlikely that such a simple system ever exists in the normal embryo. Any biological system embodies a large number of biochemical processes carried on in close relationship to one another, and a change in one process indirectly affects all the rest. It would not be possible, therefore, to build up a gradient in the concentration of any one substance without affecting the concentration of a number of others, whether these are referred to as 'evocators' or not. The only satisfactory explanation of regional differentiation possible at present is one which assumes the existence of a number of inter-related gradients. Spiegelman (1945) has recently given an interesting implementation of this idea, showing mathematically that differentiation could be controlled and limited by the competitive interaction of several biochemical processes. In any case, as Waddington has pointed out (1950), it is clear that one must allow the existence of a number of different specific evocators in order to explain the apparent specificity of some foreign tissues in inducing only certain types of nervous tissue (Chuang, 1939; Toivönen, 1949; Yamada, 1950).



Text-fig. 5. Logarithmic curve to express evocator concentration gradient within archenteron roof.

Although the present results have given no grounds for rejecting the popular idea that gradients are responsible for regional differentiation, it has emphasized the considerable regulatory powers which must exist in the organizer cells, in order to re-establish such gradients after a complete rearrangement of its cells.

SUMMARY

A series of explantation and implantation experiments has been carried out in order to discover if the amphibian organizer is able to induce normally differentiated nervous tissue after disaggregation and reaggregation of its cells.

Explants

1. In twenty-one control explants of ectoderm alone, no neural tissue was formed. This made it certain that the inductions in all the experiments were due entirely to the organizer tissue.

2. From a small number of explants of early gastrula dorsal lip into early gastrula ectoderm it was confirmed that, as Holtfreter has claimed (1944*b*), the regional inducing power of this tissue is in no way inferior after disaggregation of its cells. The numbers of brain, eye and neural tube inductions (Table 1) did not differ significantly as between experiments and controls, and these tissues were often asymmetrical in both series.

3. The archenteron roof from the late gastrula also showed no deterioration in regional inducing powers after disaggregation of its cells. When the percentages of brain, eye and neural tube inductions were compared as between experiments and controls, no significant difference was evident.

4. Combinations of three anterior, three middle or three posterior thirds of the archenteron roof in a control series gave different frequencies of brain, eye and neural tube inductions (Table 4). However, these regional differences were no longer apparent when the separate thirds had been disaggregated before explantation (Table 5). This was apparently due to a reduction of the number of brain inductions by the two anterior thirds.

5. Since no regional differences were detectable in control explants of single thirds of the archenteron roof (Table 3), there seems to be some size factor limiting the range of inductions in small explants. The influence of the quantity of inducing tissue is discussed in the light of Marx's (1925), Dalcq & Lallier's (1948) and Lopaschov's (1935) results.

6. Owing to the frequent asymmetry and the incomplete differentiation of the nervous tissue in explants, some implantation experiments were also carried out. Disaggregated dorsal lip tissue from an early gastrula was implanted into a ventral position in an early gastrula host. It appeared to induce just as successfully as the normal dorsal lip. The secondary embryos of both experiments and controls were usually incomplete. In the experiments they were more complete if they lay near the host's axis, and if the implanted tissue had invaginated for the maximum distance. The possible reasons for this relationship are discussed.

7. In the secondary embryos, the formation of symmetrical, paired somites was always associated with the presence of an archenteron and a notochord. Explants containing no endoderm never form an archenteron, and in the present experiments they also only rarely formed somites.

8. It is concluded that these results confirm Holtfreter's findings by showing that the complete organizer, even at the late gastrula stage, can differentiate normally itself, and can induce regionally differentiated nervous tissue, after its cell arrangement has been upset by disaggregation. But the results of series 4 show that specific regional properties are no longer demonstrable in separate thirds of the archenteron roof after this treatment.

9. Comparison of explants and implants leads to the conclusion that the completeness and symmetry of all inductions depends on the final layout of the organizer tissue when it begins to induce. The possible influence of the host endoderm on this layout is discussed.

10. It has been shown that all the quantitative data can be explained by postu-

lating the existence of an anterior-posterior gradient in the concentration of a single evocator. Although results of other workers show that qualitatively different inducing substances must exist, there is no reason for rejecting the idea that regional differentiation depends partly on quantitative differences.

I wish to thank Prof. C. H. Waddington, F.R.S., for suggesting this problem, for supervising the work, and for his helpful criticism of the manuscript. I am grateful also for laboratory facilities enjoyed at the Institute of Animal Genetics, Edinburgh.

In addition, I should like to thank Mr D. Roberts and Mr D. Pinkney for help in preparing the illustrations.

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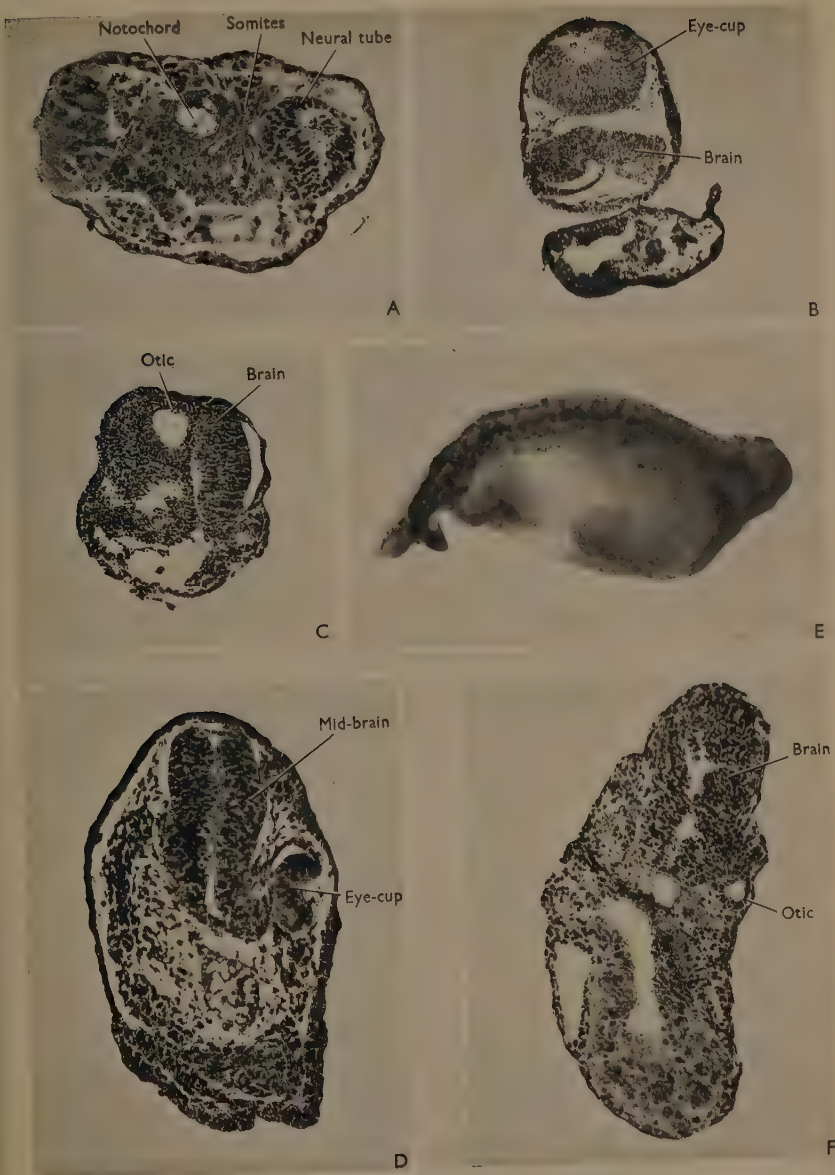

DEUCHAR—AMPHIBIAN ORGANIZER TISSUE AFTER DISAGGREGATION OF ITS CELLS IN ALKALI

EXPLANATION OF PLATE 2

Fig. A. Asymmetrical neural tube induced by explanted dorsal lip. Neural wall thickened on side next to somite tissue.

Fig. B. Small piece of brain with single eye, induced by an explant of reaggregated archenteron roof cells.

Fig. C. Asymmetrical brain and single otic capsule induced by an explant of reaggregated archenteron roof cells.

Fig. D. Symmetrical brain with incipient eye cup, induced by explanted posterior archenteron roof tissue (control).

Fig. E. Embryonic axis resulting from implantation of reaggregated dorsal lip cells into the ventral half of an early gastrula.

Fig. F. Transverse section through otic region of embryonic axis similar to the one in fig. 5.

COUNTING LIVE AND DEAD BULL SPERMATOZOA

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INTRODUCTION

The experiments described in this paper were designed to provide information, similar to that which exists in the field of haemocytometry, which will enable an experimenter to estimate the proportions of live and dead spermatozoa in a suspension and to control the precision of his estimates. In the case of blood or sperm density counts, the original suspension is diluted a known number of times and a count is made of the number of cells in a known volume of the diluted suspension. The total number counted, N, may be regarded as one sample from a series whose estimated mean is N. As is well known, the standard error of N is approximately equal to $\sqrt{N}$ in such circumstances. The position is not quite so simple in the case under consideration, as the population is composed of two classes, live and dead spermatozoa, instead of one class, erythrocytes or spermatozoa. The objects of this paper are twofold: first, to provide 'instructions' in tabular form for estimating, with a known precision, the proportion of live and dead spermatozoa in a suspension; and secondly to explain the analytical basis for these 'instructions'. As some readers may be interested in the first object, but not in the second, the statistical arguments have been put in one section, Analysis, which those who are mainly concerned with practical considerations need not read.

When bull spermatozoa are put into a mixture of nigrosin and eosin, those spermatozoa which were dead in the original sample or those which, through faulty technique, were killed by being put into the nigrosin and eosin, take up the stain and appear red under the microscope. Those which were alive in the original sample and were not killed by the staining procedure remain uncoloured. Provided that precautions are taken to prevent the spermatozoa being killed by immersion in the stain, the nigrosin-eosin technique provides a method of estimating the proportions of live and dead spermatozoa in a sample of semen. The live-dead staining technique has been developed and used by a number of workers, such as Lasley, Easley & McKenzie (1942), Emmens (1947), Crooke & Mandl (1949) and Hancock (1951). No systematic examination of the precision of estimates based on this technique has so far been made, though some of the workers mentioned above investigated it to a limited extent.

METHOD AND RESULTS

The counts of live and dead bull spermatozoa were made by Miss P. Hennessy (A.R.C.) and Miss G. E. Bending (M.R.C.). The former's experience of the live-dead staining technique was considerable while the latter's was restricted to some twenty previous experiments. Each counted the number of live and dead spermatozoa in samples from two different classes of semen, class 1 being undiluted and class 2 being semen diluted four times with Ringer-phosphate-fructose solution. The examination of undiluted semen was undertaken because of its importance in artificial insemination; the examination of diluted semen was undertaken because of the number of physiological experiments in which diluted semen is used. A fourfold dilution of bull semen is about as much as can safely be made without encountering dilution effects when protective substances such as egg-yolk are not included in the diluent. The plan of the experiment is shown in Table 1. This design was followed with both classes of semen. Each of the five subsamples was obtained from the original sample with a different Pasteur pipette (terminal diameter about 1 mm.) and one drop of semen was added to eight drops of the staining mixture which consisted of 30 g. nigrosin (Gurr) 5 g. eosin (Gurr) and 300 ml. dist. H_2O. A smear was made on a microscope slide using one drop of the mixture and mounted in DPX. In the case of diluted semen, the ratio of semen to stain was appropriately adjusted. Each operator prepared her own two classes from the original semen, and from these, her own sub-samples and slides. The results of these experiments are given in Table 2.

Table 1

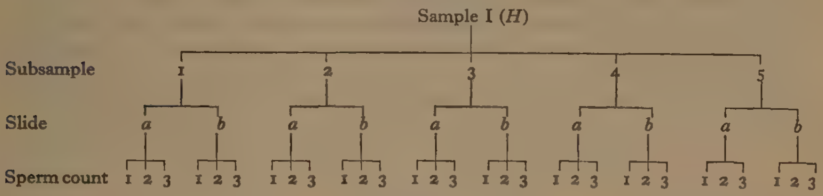

Table 2

		Miss Hennessy	Miss Bending
I, undiluted semen	No. of spermatozoa counted	6000	6720
	Mean percentage dead	11·2	9·6
	Standard error of mean (%)	0·48	0·41
II, diluted semen	No. of spermatozoa counted	6000	5953
	Mean percentage dead	10·5	10·2
	Standard error of mean (%)	0·50	0·35

Note: the differences between the standard errors of the two operators are not significant.

ANALYSIS

Samples from a population in which all the members have independently a constant chance of belonging to one of two mutually exclusive classes, as in the case of live and dead spermatozoa, will have a binomial distribution. If the mean proportion of dead spermatozoa is d, the variance of an estimate of this proportion, based on a count of a total of n spermatozoa, will be $d(1-d)/n$. The variance can be made independent of d by applying the angular transformation to the percentages of dead spermatozoa found in the various counts (Fisher & Yates, 1948, Table 12). The variance of the transformed variable is $821/n$ irrespective of the proportion dead. A series of transformed and weighted observations on one sample will have an approximately normal distribution with variance 821. The χ^2 test may therefore be applied to compare the variations between observations with those expected in random sampling. In the case of a hierarchical set of observations, such as is obtained in these experiments, the final value of χ^2 may be subdivided so that the various stages in the experiment can be examined for deviations from random sampling.

Table 2 shows that the difference between diluted and undiluted semen, both as regards the mean percentage dead and the variance estimate, is insignificant. This holds for both operators. This result was surprising as it was expected that the process of dilution would increase the proportion of dead spermatozoa. If, on the other hand, the two operators are compared in respect of their estimates of the mean percentage dead, the difference between the two values, pooling the results for diluted and undiluted semen, may be expressed as a χ^2 of 6·698 on 1 degree of freedom, which is significant at the 1 % level. The variance based on Miss Hennessy's counts was larger than expectation at the 0·1 % level of significance, while that based on Miss Bending's counts was close to the theoretical value for random sampling. This difference in the variance estimates, which may be expressed as a variance ratio of 1·72, distributed as F on 59, 56 degrees of freedom, is significant at the 5 % level. This heterogeneity of the two operators is discussed later.

Table 3. *Subdivision of the total χ^2 according to stages in the sampling procedure*

	Miss Hennessy			Miss Bending		
	χ^2	D.O.F.	Significance	χ^2	D.O.F.	Significance
Between counts	52·110	40	—	28·745	38	—
Between slides	16·055	10	—	6·002	9	—
Between subsamples	29·500	8	0·1 %	19·056	8	1 %

D.O.F., degrees of freedom.

The overall χ^2 expressing the variation between single counts for each operator may be subdivided so as to examine the variation between counts on one slide, the variation between slides in one subsample, and the variation between subsamples derived from one sample, each of which relate to a particular stage in the sampling procedure (Table 1). This is done in Table 3 from which it will be seen that the variations between different counts on particular slides, and between counts on different slides from one subsample are consistent with random binomial variations, in the case of each operator. This is not, however, so at the subsample level, a fact which is responsible for the heterogeneity referred to in the previous paragraph. This means that the number of spermatozoa which must be counted to obtain a given precision goes down as the number of subsamples taken from the original semen is increased. Tables illustrating this point are given in the next section.

The precision of the estimate of the proportion of dead spermatozoa is conveniently expressed in terms of the coefficient of variation of the percentage dead, C.V. $= 100s/m$, where m is the mean percentage dead and s is the standard error of the mean. The standard error depends on the number of spermatozoa counted and on the way this number is derived from subsamples and slides. Because of the heterogeneity between subsamples *only limited precision* can be achieved through counts based on one subsample, i.e. one drop of semen. The variance within one subsample is $821/nk$ (transformed estimate of proportion dead), where the estimate is based on n counts of k spermatozoa. The variance between subsamples, σ^2, may be estimated from the 'between subsamples' term in the analysis of variance. The total variance of an estimate based on one subsample is σ^2+821/nk and of an estimate based on the mean of p subsamples $p^{-1}(\sigma^2+821/nk)$; from which it is obvious that if $p=1$, the variance cannot fall below σ^2, however many spermatozoa are counted, i.e. if $nk \rightarrow \infty$. If the number of counts is constant, the variance is minimized when one count is made from each subsample. Since p, n and k can be varied independently, the experimenter can alter them while maintaining the precision constant, to satisfy some further condition such as dislike of making more than two smears, counting spermatozoa on more than one slide, etc. Examples are given in the next section.

When single smears are made from each of p subsamples, k spermatozoa being counted on each smear, the estimate of the proportion dead is

$$\hat{P}=\frac{1}{pk}\sum_{i=1}^{n} r_i$$

where r_i is the number dead in the ith count. $\hat{P}$ is related to ϕ, the angular variable, by the relationship $\hat{P}=\sin^2\phi$. $V(\hat{P})$ is determined from the known form of $V(\phi)$ by application of the relationship $V[f(x)]=(df/dx)^2V(x)$. This enables the coefficient of variation to be computed, as in Table 4.

PRACTICAL INSTRUCTIONS

Table 4 shows how the coefficient of variation of an estimate of the percentage of dead spermatozoa in a suspension varies according to the percentage dead and the total number of spermatozoa counted. The coefficient of variation is defined as C.V. $=100s/m$, where m is the percentage or mean percentage dead and s is the standard error. Suppose, for example, that a mean percentage dead of 10 is associated with a coefficient of variation of 20%. This means, roughly, that if the true percentage dead is not between 6 and 14, there is less than a 5% chance of getting an estimate equal to 10 for it in this hypothetical experiment. The number of spermatozoa which must be counted to achieve a desired precision goes down as the number of subsamples taken from the original semen goes up, and, therefore, as the number of smears which the experimenter makes increases. Consequently, Table 4 is expressed in terms of hundreds of spermatozoa *counted per smear from different subsamples*. Suppose that the percentage of dead spermatozoa is 10, and 100 spermatozoa are counted on one smear. Reference to Table 4 shows that the coefficient of variation is 32%; the percentage dead is therefore 10 ± 6·4 (at the 95% level). If, however, two smears are made, by taking two subsamples, i.e. drops, from the parent suspension, and two lots of 100 spermatozoa are counted, one on each smear, the coefficient of variation is reduced to 23% making

Table 4. *Change in coefficient of variation according to the percentage dead, P, and the total number of spermatozoa, N, counted (in hundreds)*

Each hundred spermatozoa are counted on a separate smear prepared from a separate subsample so that, 300, for example, means 100 spermatozoa on each of three smears. If the percentage dead is more than 50, use the percentage of live spermatozoa for calculations and the same table.

P (%) N	1	2	5	10	15	20	25	30	40	50
100	107	75	47	32	26	22	18	17	13	11
200	76	53	33	23	18	15	13	12	9	8
300	62	44	27	19	15	12	11	10	8	6
400	53	38	24	16	13	11	9	8	7	5
500	48	34	21	14	12	10	8	7	6	5
600	44	31	19	13	11	9	7	7	5	4
700	40	29	18	12	10	8	7	6	5	4
800	38	27	17	11	9	8	6	6	5	4
900	36	25	16	11	9	7	6	6	4	4
1000	34	24	15	10	8	7	6	5	4	3
1100	32	23	14	10	8	7	5	5	4	3
1200	31	22	14	9	7	6	5	5	4	3

Table 5. *Change in coefficient of variation according to the percentage dead, P, and the total number of spermatozoa, N, counted on one smear*

P (%) N	1	2	5	10	15	20	25	30	40	50
100	107	75	47	32	26	22	18	17	13	11
200	81	57	36	25	19	16	14	12	10	8
300	70	49	31	21	17	14	12	11	9	7
400	64	45	28	19	15	13	11	10	8	6
500	60	42	26	18	14	12	10	9	7	6
600	57	40	25	17	14	12	10	9	7	6
700	55	39	24	17	13	11	9	9	7	6
800	54	38	24	16	13	11	9	8	7	5
900	52	37	23	16	13	11	9	8	6	5
1000	51	36	23	16	12	10	9	8	6	5
1100	50	36	22	15	12	10	9	8	6	5
1200	50	35	22	15	12	10	8	8	6	5

Table 6. *Change in coefficient of variation according to the percentage dead, P, for various groupings of sperm counts*

p, number of smears from separate subsamples; N, number of spermatozoa counted per smear.

p	P (%) N	1	2	5	10	15	20	25	30	40	50
2	200	57	40	25	17	14	12	10	9	7	6
	300	50	35	22	15	12	10	8	8	6	5
	400	45	32	20	14	11	9	8	7	6	5
	500	42	30	19	13	10	9	7	7	5	4
	600	41	29	18	12	10	8	7	6	5	4
3	200	47	33	21	14	11	9	8	7	6	5
	300	40	29	18	12	10	8	7	6	5	4
	400	37	26	16	11	9	7	6	6	5	4
4	200	40	29	18	12	10	8	7	6	5	4
	300	35	25	15	11	8	7	6	5	4	4
5	200	36	26	16	11	9	7	6	6	4	4
6	200	33	23	15	10	8	7	6	5	4	3

the estimate of the percentage dead 10 ± 4·6% (at the 95% level). To achieve 10 ± 2% in this example, ten smears must be made and 100 spermatozoa must be counted on each smear. If the percentage dead is higher, the labour involved in achieving some desired precision is less. Some experimenters may find it more convenient to make counts on only one smear, though this practice may be dangerous because it provides no evidence of faulty technique. Table 5 shows how many spermatozoa must be counted in this case, at various levels of percentage dead, to achieve any required coefficient of variation. Table 6 shows how many spermatozoa must be counted for a given precision if only a small number of smears are made.

If an experimenter wants to achieve some particular precision, irrespective of the proportion of dead spermatozoa present, he should stop after counting 100 spermatozoa and make a rough estimate of the percentage dead. Having got this rough estimate, reference to Tables 4 or 5 will show how many more must be counted to obtain the required precision.

SUMMARY

1. An investigation has been made into the accuracy of estimates of the proportions of dead spermatozoa in diluted and undiluted bull semen, using the nigrosin-eosin live-dead staining technique.

2. Two operators counted 24,673 spermatozoa, in lots of approximately 200, on smears derived from a number of subsamples taken from the same ejaculate.

3. The variations between different counts made on one smear were consistent with random sampling from a binomial population; the same applied to variations between slides made from one subsample; the variation between subsamples were, however, larger than would have been expected on the basis of random sampling.

4. Undiluted semen and semen diluted 1/4 with Ringer-phosphate-fructose solution had the same mean percentage dead and precision of estimation.

5. Tables are given showing how the precision of an estimate of the proportion of dead spermatozoa in a sample varies according to the percentage of dead spermatozoa, the total number of spermatozoa counted and according to the number of subsamples taken from the original ejaculate, each subsample being used for making counts.

We are much obliged to Prof. Sir Ronald Fisher, F.R.S., for advice. One of us (R.) is indebted to the Medical Research Council for the provision of a laboratory assistant.

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THE SPERMATOZOA OF STERILE BULLS

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(With Plates 3 and 4)

INTRODUCTION

This paper is concerned with a morphological abnormality in the spermatozoa of certain sterile bulls and the identification of this abnormality when specimens are prepared for microscopical examination.

In certain cases sterility in the bull can be associated with the presence of an abnormal acrosome, the abnormality taking the form of an eccentrically placed thickening of this structure (Teunissen, 1946; Blom, 1948; Hancock, 1949; Rollinson & Makinson, 1949; Bretschneider, 1950). A number of photographs of it are given later in this paper. In the United Kingdom it has been recognized in seventeen Friesian bulls, sixteen of which were closely related. The clear-cut nature of the defect and its transmission by apparently normal parents suggests that it is due to a recessive gene.

Recent work on the preparation of spermatozoa for microscopical examination shows that considerable care must be taken to preserve the morphological characteristics of the living spermatozoa before fixation. This question has been discussed in detail in a recent paper (Hancock, 1952), and here it is only necessary to say that unless special precautions are taken to avoid damage from such causes as sudden cooling, fixed preparations are morphologically very different from living ones. When examining spermatozoa for evidence of the genetical defect referred to above, it is essential to take precautions to avoid accidental damage if the correct incidence of this defect is to be estimated.

The acrosome is situated at the anterior half of the head. It is a compact refractile structure which in the living spermatozoon is closely applied to the surface of the sperm head. After maltreatment or in certain pathological cases, it may appear as a fragile loose envelope. Blom (1945) calls this structure the *galea capitis*, but for reasons discussed in detail elsewhere the word 'acrosome' is preferable.

The spermatozoa of bulls with this genetical defect may appear to be physiologically normal (Rothschild, 1950), which makes accurate morphological information about them of special importance. The experiments described in this paper were designed to investigate the effects of post-mortem changes, caused by various treatments, on the morphology of spermatozoa with this defect.

When spermatozoa are examined in a dry film under the microscope they are of course dead. Some of these may have been dead originally, in the sample of semen;

others may have been killed during the treatment of the material before the film was prepared, while others may have been alive until the moment when the film was fixed or dried. In investigating the microscopical or sub-microscopical morphology of spermatozoa, whether originally normal or abnormal, a clear distinction must be made between these three classes; otherwise, as has happened in the past, it is difficult to decide which morphological structures were originally present, which were induced by the treatment prior to examination, and which were masked by the preparatory treatment. In order to maintain these distinctions special meanings have been given to certain words used in this paper. A spermatozoon is (1) *living* if it is alive when it is examined with the microscope; a spermatozoon is (2) '*live*' if it has been alive up to the moment of fixation (or drying). A spermatozoon may have been (3) *originally dead* when the sample was collected or it may have been (4) *killed* during the preparatory treatment, e.g. by 'temperature shock'. In cases where it is unnecessary or impossible to distinguish between (3) and (4) the word (5) *dead* is used. When a spermatozoon shows the eccentrically placed thickening of the acrosome, with which this paper is concerned, it is referred to as (6) *abnormal*.

MATERIALS AND METHODS

The semen of three Friesian bulls (numbered 1, 2 and 3), all with clinical histories of virtual sterility, was collected in the usual way and stained with nigrosin-eosin, Giemsa and Heidenhain's iron haematoxylin, as described in an earlier paper in this *Journal* (Hancock, 1952). Studies of dead spermatozoa were made on samples killed by rapid cooling as previously described. The procedure for electron micrography was the same as before, except that the semen was fixed by dilution with an equal volume of neutral formal saline (5% formaldehyde) and washed after fixation for 1 hr. The electron micrography was carried out by Mr R. W. Horne, Cavendish Laboratory, University of Cambridge.

The percentage of morphologically abnormal forms was determined from counts of four hundred spermatozoa per sample.

RESULTS

In smears of abnormal spermatozoa, stained by the nigrosin and eosin method, the 'live' spermatozoa (which do not take the stain) are seen to possess a highly refractile area at the distal end of the acrosome; in dead spermatozoa (which do take the stain) there is little evidence of this abnormality. The characteristic appearances of 'live' and dead spermatozoa are seen in Pl. 3 *a*, *b*. Both these preparations were made from the same ejaculate, (*a*) being stained at 30° C. and (*b*) being stained at 10° C. and thus subjected to temperature shock. In (*a*) the abnormality is clearly seen in all four spermatozoa; in (*b*) it is seen in one spermatozoon which is unstained and therefore 'live' whereas it is not seen in the other two which have taken the stain and were presumably killed by temperature shock.

Differential counts made from smears prepared after staining at different temperatures showed that the proportion of clearly abnormal spermatozoa declined as the temperature difference between semen and stain was increased. This decline

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was associated with a decrease in the percentage of spermatozoa which survived the temperature shock imposed during staining. The results of one experiment, using the semen of bull 1, are summarized in Table 1.

Studies of unfixed material with the phase-contrast microscope confirmed that the appearance of the abnormal acrosome is modified by the death of the spermatozoon. Using this technique the acrosome appears as a sharply defined dark refractile area in the living spermatozoon; but it is quite inconspicuous in dead cells. Its appearance in living and dead spermatozoa viewed by this method is shown in Pl. 3 *c*, *d*.

Table 1. *The effect of stain temperature on the staining affinity of bull spermatozoa and on the abnormal acrosome* (*nigrosin-eosin*)

Stain temperature (° C.)	No. of spermatozoa in 200				Mean (%)	
	Smear 1		Smear 2			
	L	A	L	A	L	A
30·0	130	95	131	82	65·25	44·25
20·5	75	69	77	55	38·0	31·0
15·0	28	24	36	17	16·0	10·25
2·5	1	5	0	1	0·25	1·5

Live unstained, L; visibly abnormal, A, temperature of semen, 30° C.

In haematoxylin-stained smears of 'live' spermatozoa, the abnormality is seen as an intensely stained area of the acrosome. It is less conspicuous in dead spermatoza, but is not infrequently recognizable as a fragile prominence at the distal border. 'Live' and dead spermatozoa in smears stained with Giemsa or with indian ink show no marked difference in microscopical appearance. Pl. 3 *e*, *f*, show smears of dead spermatozoa stained by these techniques. Electron micrographs of the abnormality in 'live' and dead spermatozoa are shown in Pl. 4 *a*, *b*.

The proportion of visibly abnormal spermatozoa was determined in 'live' and killed samples of seven ejaculates from two other Friesian bulls (bulls 2 and 3), using a number of different techniques. The results are summarized in Table 2.

Statistical analysis, for which I am indebted to Mr R. C. Campbell of this Unit, shows that the abnormality is equally apparent in 'live' and dead spermatozoa stained by the indian ink and Giemsa techniques. This is not, however, the case when the nigrosin-eosin or phase-contrast techniques are used, the abnormality being invisible in dead spermatozoa prepared by these methods. The same applies in the case of haematoxylin-stained material though the relationship between the percentage of 'live' spermatozoa, and the percentage of visibly abnormal spermatozoa is not the same as when the nigrosin-eosin and phase-contrast techniques are used.

These relationships also apply when the data for the 'live' and killed samples are studied separately. There is therefore no evidence that the cytological changes which occur in spermatozoa killed by rapid cooling ('temperature shock') differ from those which distinguish living from dead spermatozoa in freshly ejaculated semen.

Table 2. *Comparison of different methods of examining the abnormal acrosome in 'live'* (L) *and dead* (D) *spermatozoa*

Bull	Ejaculate	Sample	Live (%)	Abnormal (%)				
				Nigrosin-eosin	Phase contrast	Iron-haema-toxylin	Giemsa	Indian ink
2	1	L	90	91	81	—	—	—
		D	7	6	7	—	—	—
2	2	L	79	80	75	85	99	—
		D	13	15	14	52	99	—
2	3	L	67	70	62	92	95	96
		D	5	2	1	21	94	95
2	4	L	77	72	62	72	97	96
		D	49	45	38	48	96	95
2	5	L	81	77	67	80	95	95
		D	9	8	3	15	91	94
3	1	L	62	62	—	—	95	95
		D	6	7	—	—	95	97
3	2	L	50	52	—	—	92	97
		D	6	6	—	—	91	96

The abnormal features of living and dead spermatozoa are compared in the column headed Phase contrast.

DISCUSSION

Diagnosis of sterility in bulls whose spermatozoa exhibit this acrosomal abnormality can at present only be based on microscopical examination. After the death of the spermatozoa, changes occur in the acrosome which render the abnormality indistinct or unrecognizable by certain microscopical techniques. Smears of killed spermatozoa stained with nigrosin and eosin or with Heidenhain's iron haematoxylin show little evidence of the abnormality: it is also indistinct in dead spermatozoa examined with the phase-contrast microscope. In smears stained by the Giemsa and indian ink techniques the abnormality is equally evident in both 'live' and dead spermatozoa.

Freshly ejaculated semen contains a variable proportion of originally dead spermatozoa; living spermatozoa are readily killed by such treatments as rapid cooling. Of the techniques used only the Giemsa and indian ink methods will enable an accurate estimate to be made of the proportion of abnormal spermatozoa in a sample.

SUMMARY

1. Sterility in certain Friesian bulls is associated with a specific morphological abnormality affecting the acrosomes of their spermatozoa.

2. This abnormality is visible in living spermatozoa and is also visible in fixed spermatozoa when precautions are taken to preserve the morphology of the cell.

3. If such precautions are not taken, and the spermatozoa are subsequently stained by the nigrosin-eosin method, or with Heidenhain's iron haematoxylin, or

when such spermatozoa are examined with the phase-contrast microscope, the abnormality is inconspicuous.

4. These difficulties do not arise when either the Giemsa stain or indian ink is used.

I wish to thank Mr J. Smiles, National Institute for Medical Research, Mill Hill, London, for the photomicrographs in Pl. 3 *a*, *b*, Mr A. V. Guntrip for those in Pl. 3 *c–f*, and Miss P. J. Hennessy for her technical assistance.

The bulls were made available through the collaboration of Mr E. J. Simmons, M.R.C.V.S., Mr B. V. Sinclair, M.R.C.V.S., and Mr G. F. Smith, M.R.C.V.S., to whom I am grateful.

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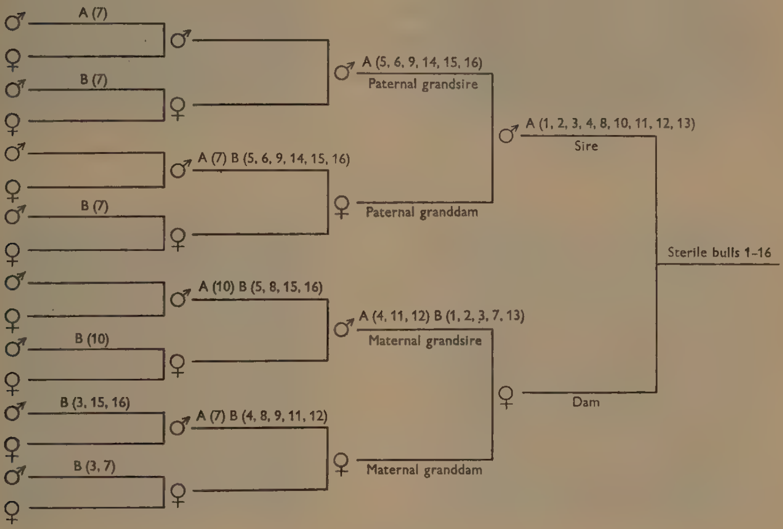
APPENDIX

Although this investigation was primarily concerned with the morphology of spermatozoa it has brought to light a certain body of information which bears upon the inheritance of the abnormality and may be of interest to geneticists.

An examination of the pedigrees of the sixteen sterile bulls shows that they are all related to two bulls which we will call A and B. The relationships are summarized in a chart (Text-fig. 1). Many people find this presentation difficult because they try to interpret Text-fig. 1 as a pedigree chart of the orthodox type. To understand how the chart is to be read the reader is advised simply to work through the following examples in the exact order indicated in the instructions and omitting none of the steps.

Take first the relationship of *sire*. Find the line labelled 'sire' on the chart. Note that associated with 'sire' are the symbols A (1, 2, 3, 4, 8, 10, 11, 12, 13). This means that bull A was the sire of the sterile bulls 1, 2, 3, 4, 8, 10, 11, 12 and 13. Next, take the relationship of *maternal grandsire*. Find the line labelled 'maternal grandsire' on the chart and note that it bears the symbols A (4, 11, 12) B (1, 2, 3, 7, 13). This means that bull A was the maternal grandsire of sterile bulls 4, 11 and 12 and that bull B was the maternal grandsire of sterile bulls 1, 2, 3, 7 and 13. Next find the line at the top left-hand corner of the chart which is unlabelled but bears the symbols A (7). The proper label for this line is 'father of the father of the paternal grandsire', as the chart indicates. The symbols A (7) in association with this line mean that bull A was the father of the father of the paternal grandsire of sterile bull 7. Now search the chart and note all the positions in which the figure 7 occurs. There are three positions where 7 occurs in brackets after A and four positions where 7 occurs in brackets after B.



Text-fig. 1.

The three positions where 7 occurs in brackets after A indicate that bull A was:

(i) the father of the father of the paternal grandsire,
(ii) the father of the paternal granddam,
(iii) the father of the maternal granddam,

of sterile bull 7.

The four positions where 7 occurs in brackets after B indicate that bull B was:

(iv) the father of the mother of the paternal grandsire,
(v) the father of the mother of the paternal granddam,
(vi) the father of the mother of the maternal granddam,
(vii) the maternal grandsire,

of sterile bull 7.

It should now be possible for the reader to take any other sterile bull, say sterile bull 5, find the positions where the figure 5 occurs on the chart and thereby discover where bulls A and B occur in the family history of sterile bull 5.

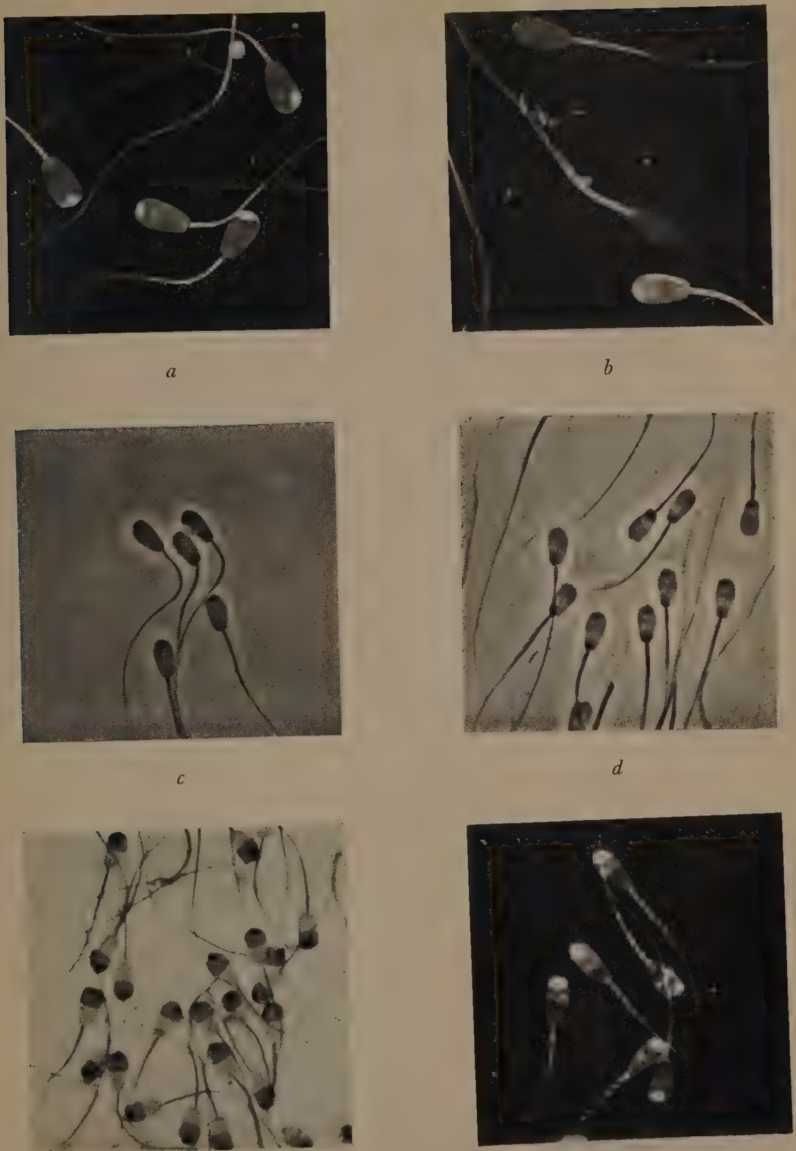
EXPLANATION OF PLATES

PLATE 3

(*a*) 'Live' spermatozoa showing abnormal acrosome. Nigrosin-eosin. × 950.
(*b*) One 'live' and two dead spermatozoa showing altered features of abnormal acrosome in dead spermatozoa. Nigrosin-eosin. × 950.
(*c*) Living spermatozoa showing abnormal acrosome. Phase-contrast. × 800.
(*d*) Dead spermatozoa showing altered features of abnormal acrosome. Phase-contrast. × 800.
(*e*) Dead spermatozoa showing abnormal acrosome. Giemsa. × 680.
(*f*) Dead spermatozoa showing abnormal acrosome. Indian ink. × 680.

PLATE 4

(*a*) 'Live' spermatozoon showing abnormal acrosome. Electron micrograph. Shadowed gold-palladium. × 14,000.
(*b*) Dead spermatozoon showing abnormal acrosome. Electron micrograph. Shadowed gold-palladium. × 16,500.

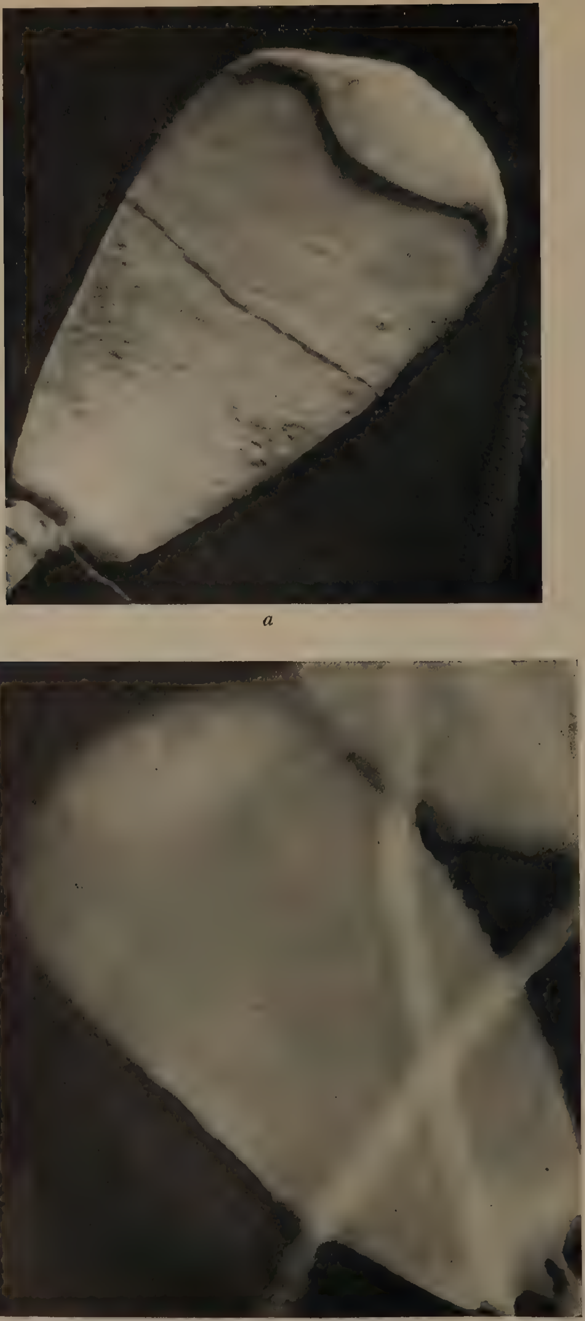


a *b*

c *d*

e *f*

HANCOCK—THE SPERMATOZOA OF STERILE BULLS



a

b

HANCOCK—THE SPERMATOZOA OF STERILE BULLS

THE FERTILIZATION REACTION IN THE SEA-URCHIN

THE INDUCTION OF POLYSPERMY BY NICOTINE

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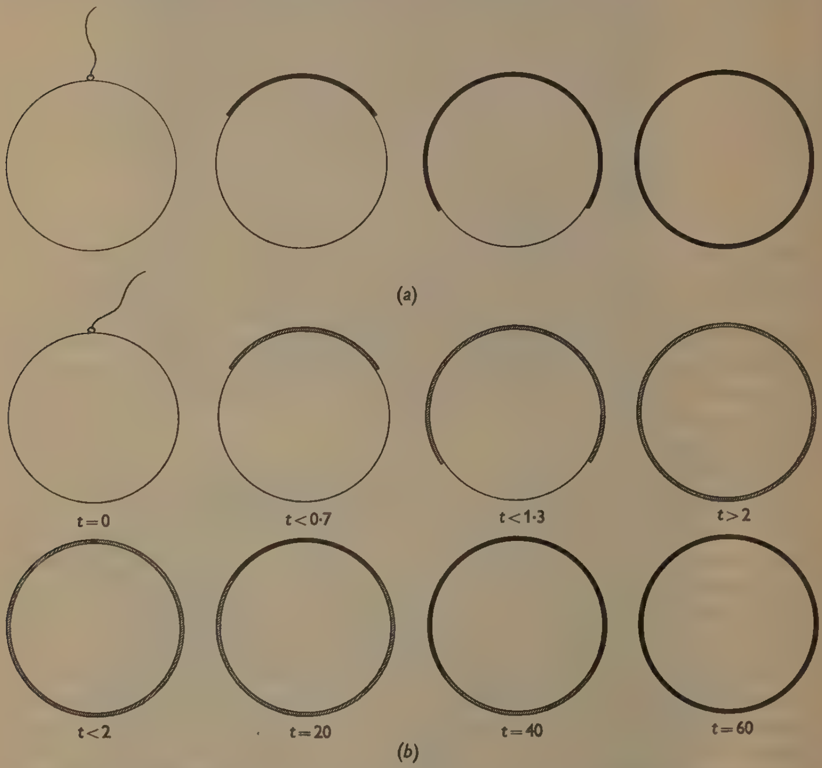
(With Plate 5)

INTRODUCTION

Monospermy is one of the mechanisms which ensures that male and female hereditary characteristics are present in the offspring in approximately equal quantities. There are three main methods by which this is achieved: first, by the neutralization of supernumerary spermatozoa after they have entered an egg; secondly, by preventing supernumerary spermatozoa from entering an egg; and thirdly, by the death of a polyspermic embryo. The first of these methods has been observed in the eggs of birds, amphibians and possibly some mammals; the third requires no comment, while the second is the subject of this paper and may be defined as follows: when a spermatozoon fertilizes an egg a change occurs in the structure of the egg surface which prevents re-fertilization. This change in surface structure, the block to polyspermy, is initiated by the fertilizing spermatozoon and spreads over the egg surface from the point of sperm attachment. It may be likened to a skin which is impermeable to spermatozoa, spreading or expanding over the egg surface (Text-fig. 1 *a*). Recent experiments show that this model is over-simplified and that the skin, instead of being totally impermeable to spermatozoa (or 'black'), from the moment the fertilizing spermatozoon causes it to appear, starts by being relatively impermeable to spermatozoa (or 'grey'), as in Text-fig. 1 *b*. Later on, the egg surface becomes completely impermeable to supernumerary spermatozoa, when the block to polyspermy is complete. These conclusions about the block to polyspermy are based on a series of experiments (Rothschild & Swann, 1949–52) which are summarized immediately below. When a sea-urchin egg is fertilized, a change in the structure of the egg surface, the cortical change, spreads over the cortex from the point of attachment of the fertilizing spermatozoon. The conduction time of this change is of the order of 20 sec. Calculations, which involve treating spermatozoa as gas molecules bombarding a sphere (the validity of which is discussed later), show that if this cortical change is the block to polyspermy, the probability of a successful sperm-egg collision, in the sense that fertilization follows the collision, must be of the order of 1 in 160. This question was investigated in another series of experiments in which it was shown that though the probability of a successful sperm-egg collision was markedly less than one, it was not low enough, i.e. 1/160, to support the hypothesis that the cortical change was the block to polyspermy. At the same time, there was other evidence showing that the block to polyspermy was a comparatively slow reaction, taking something of the order of seconds to cover the egg surface. These apparent contradictions were resolved when the block to polyspermy was shown to be diphasic, in the sense that it consisted of a fast component with a conduction time of less than 2 sec. and a slow component which takes about 60 sec. to cover the egg surface and make it completely impermeable to spermatozoa. After the fast phase of the block to polyspermy is complete the chance of re-fertilization is reduced by a factor of about 20.



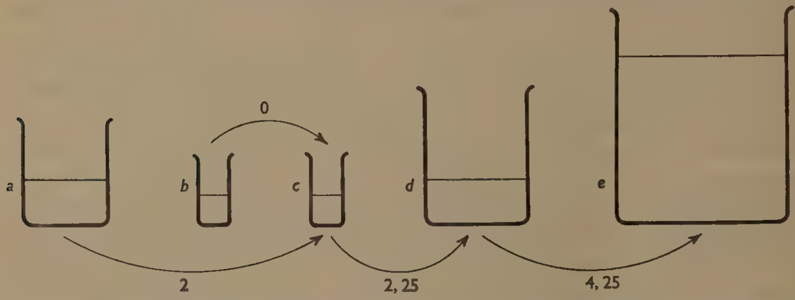
Text-fig. 1. *a*, simplified diagram of block to polyspermy. *b*, revised diagram showing rapid partial block (grey), and slow complete block (black). Time, *t*, in seconds.

The fertilization rate or parameter, α. As there are several references to α in this paper, some observations on its significance may be desirable. No recondite arguments are needed to support the contention that the chances of fertilization depend, among other things, on the speeds at which spermatozoa swim, if only because it is easy to show that spermatozoa which are normally motile, but which have been rendered motionless, cannot fertilize eggs. It has, however, proved valuable to regard a mixture of eggs and spermatozoa as a system of spheres being bombarded by ideal gas molecules, moving at known speeds. The application of kinetic theory principles to suspensions of eggs and spermatozoa revealed, as mentioned above, that the conduction time of the block to polyspermy could not be of the order of 20 sec. unless the probability of a successful sperm-egg collision was 1 in 160 or less. Furthermore, this quantitative examination of the fertilization reaction enabled an estimate to be made of the probability of a successful collision. Some biologists have nevertheless expressed doubts about the validity of using concepts borrowed from the kinetic theory of gases as an aid to understanding the fertilization reaction. These doubts are based on the idea that the expression 'sperm-egg collision' implies that if we were to take a ciné-film of a single egg in a suspension of spermatozoa, we should see numbers of marble-like or, more accurately, tadpole-like objects colliding with and bouncing off the egg surface. It is hardly necessary to say that spermatozoa do not behave in this way: almost every student of the fertilization reaction must have noticed spermatozoa stuck to the surface of an egg but unable to fertilize it. Such deviations from 'ideal' behaviour are, in fact, unlikely to make estimates of collision frequencies seriously in error; but even if we do not start with the idea of sperm-egg collisions, we are inevitably driven to some such concept when investigating the relationship between the number of fertilized eggs in a suspension and the time of contact between the eggs and spermatozoa in that suspension. When this experiment is carried out, the curve relating the proportion of fertilized eggs to the sperm-egg interaction time is found to be of the form $f = 1 - \exp(-\alpha t)$, where α is a constant and t is the interaction time (maximum, 40 sec.). If, on the other hand, we make the plausible and weak assumption that the probability of an egg being fertilized in an interval of time δt is proportional to that interval of time, the proportionality constant being denoted by α, we again arrive at the equation $f = 1 - \exp(-\alpha t)$. For purely dimensional reasons, α must be a rate or frequency, because the exponent on the right-hand side of the equation must be dimensionless. No information is lost by considering α as a fertilization rate or parameter, but if this is held to be too abstract, α can be thought of as the product of the sperm-egg collision frequency, Z, and the probability of such a collision being successful, p. The α concept does not involve consideration of such questions as the random movements of spermatozoa, chemotaxis, or the trap action of jelly; these only arise when α is expressed in terms of Z and p.

Nicotine. Pre-treatment of eggs with nicotine increases the chance of re-fertilization by a factor of about 20, when insemination is carried out at high sperm densities 10^8/ml. (Rothschild & Swann, 1950). The object of the experiments described in this paper was to find out how the nicotine treatment achieves this result. There are three or perhaps four possibilities, which could occur separately or in combination: (1) nicotine might increase the speeds at which spermatozoa swim, thereby increasing the probability of a sperm-egg collision; in other words it might increase α. (2) Nicotine might lengthen the conduction time of the block to polyspermy, thus increasing the probability of supernumerary spermatozoa fertilizing the egg before its surface became totally impermeable. (3) Nicotine might make the surface of the egg more receptive to spermatozoa. This is equivalent to increasing α, but via the egg and not the spermatozoon. We can imagine that if the surface of an egg were magnified sufficiently, it would appear to be covered with a series of incomplete but identical jig-saw puzzles. If nicotine made the consistency of these puzzles more like rubber than wood, it is not difficult to conceive that a spermatozoon might find it easier to complete one puzzle and initiate fertilization. (4) The fourth and unlikely mode of action of nicotine arises from the observation, mentioned earlier, that the conduction rate of the block to polyspermy is markedly non-uniform, there being a fast component which probably covers the egg in less than 2 sec. and a slower component which makes the egg totally impermeable to spermatozoa. As the conduction time of the cortical change is unaffected by nicotine (Rothschild & Swann, 1950) and as the fertilization membrane appears at the normal time in nicotine-treated eggs, the possibility that nicotine retards the slow component is remote.

MATERIAL AND METHODS

The gametes of *Paracentrotus lividus* were used at room temperature, 15–17° C. The jelly was not removed from the eggs and was present at the end of each experiment. Eggs and spermatozoa were allowed to interact with each other for known periods of time, functional separation being effected by the hypo-hypertonic treatment described in detail by Rothschild & Swann (1951*b*). This treatment was particularly successful with the gametes of *P. lividus*, as the transfer of the eggs from one beaker to another did not cause any cytolysis, while the spermatozoa were killed by immersion in hypotonic sea water and no re-fertilization occurred on transfer to hypertonic sea water. The sequence of operations is shown diagrammatically in Text-fig. 2. Eggs were pre-treated with sea water containing re-distilled



Text-fig. 2. Experimental procedure. The curved lines with arrows show which vessels are emptied into which. The numbers by the curved lines refer to the times of emptying, e.g. 2, 25 means that vessel *c* was emptied into vessel *d* 2 min. 25 sec. after the beginning of the experiment, which started when *b* was emptied into *c* at $t=0$. *a*, 10 ml. of sperm suspension; *b*, 2 ml. nicotine in sea water (1/1000, v/v); *c*, 2 ml. egg suspension; *d*, 90 ml. hypotonic sea water (28 %); *e*, 700 ml. sea water + 21 ml. 11 % NaCl in sea water.

nicotine (1/2000, v/v), for 2 min. before fertilization. The sperm-egg interaction time was 25 sec.

Although superficially this type of experiment appears to be simple, in practice a number of difficulties were encountered. These centred round the problem of adjusting four variables simultaneously to achieve 30–70% fertilization in the egg suspensions. The variables in question were: the concentration of nicotine in the sea water; the duration of the pre-treatment of eggs with nicotine—there is a non-linear strength-duration relationship which determines the incidence of polyspermy; the sperm-egg interaction time; and the density of spermatozoa in contact with the eggs. The only way of solving this problem was by undertaking a series of pilot experiments before each of the experiments referred to in the Results Section. It was impossible to standardize the experimental conditions once and for all because eggs differed in their susceptibility to nicotine from batch to batch, and the proportion of dead spermatozoa in a sperm suspension also varied in samples from different sea-urchins.

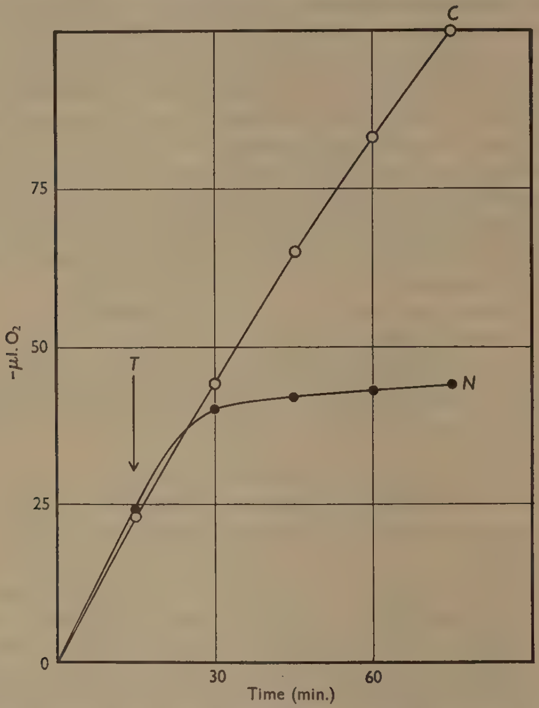
The usual controls for unfertilizable eggs, parthenogenetic activation and fortuitous fertilization at times other than 0–25 sec. were done as a matter of routine.

Measurements of sperm respiration were made in conventional Warburg differential manometers, by the direct method.

RESULTS

Effect of nicotine on spermatozoa. Visual examination showed that nicotine did not stimulate spermatozoa to increased activity, while dark ground tracks of spermatozoa in very dilute suspensions treated with nicotine confirmed the results of visual examination. These rough observations were amplified by measurements of the O_2 uptake of spermatozoa, before and after addition of nicotine to the medium (Text-fig. 3). Evidently nicotine is highly toxic to sea-urchin spermatozoa though there is a time-lag before it exerts its toxic effect. These experiments establish that nicotine does not induce polyspermy by increasing the activity of the spermatozoa, which could increase the chances of fertilization.

Probability of fertilization. Having disposed of the possibility that the activity of the spermatozoa is increased by nicotine, the eggs were tested to see if the nicotine altered their surfaces in such a way that the probability of fertilization was increased. Suppose that the sperm density in contact with the eggs and the sperm-egg interaction time are so adjusted that *not all* the eggs, but only a proportion, are fertilized at the end of the experiment. In these conditions, if nicotine were to increase the probability of fertilization, pre-treatment with nicotine should increase the proportion of eggs fertilized. The results of experiments to test this hypothesis are given in Table 1. The same number of eggs, pre-treated or not pre-treated with nicotine, were allowed to interact with the same number of spermatozoa for 25 sec. The proportion of fertilized eggs was determined after first cleavage in each case. Table 1 shows that nicotine does increase the probability of fertilization, α_N/α_C being greater than one in each case; but this increase, the average of which is 3·5, is insufficient to account for the facts of polyspermy in treated and untreated eggs, the required factor being about 20. The eggs of *P. lividus* are similar to those of *Psammechinus miliaris* regarding the incidence of polyspermy in untreated eggs (Rothschild & Swann, 1950), which means that α is of the same order in both species.



Text-fig. 3. Oxygen uptake of sea-urchin spermatozoa after addition of nicotine. Contents of flasks, 3 ml. of semen diluted 1/100 with sea water. Side-arms, curve *N*, 0·3 ml. nicotine in sea water; curve *C*, 0·3 ml. sea water. *T*, side-arm contents tipped into main compartments of flasks. Centre wells, 0·2 ml. 10% KOH and filter-papers; shaker, 90 c.p.m.; stroke, 4·0 cm.; temperature, 17° C.; gas phase, air; vessel constants, app. 1.

During these experiments certain other observations were made and deserve mention:

(1) If an egg is fertilized just at, or before, the moment of transfer to hypotonic sea water, activation of the egg occurs but division does not take place. Membrane elevation is restricted to a small blister at the point where the sperm became attached to and activated the egg. The egg nucleus swells up and moves to the centre of the egg, but no development occurs. Except for the lack of division the phenomenon is reminiscent of the pseudogamy which occurs in *Rhabditis monohystera* and *R. pellio* (Peacock, 1944). Such activated eggs are not difficult to identify, provided it is realized that uncleaved eggs without two asters or a normal fertilization membrane

have been 'fertilized'. The investigation of this phenomenon, though of interest, is not relevant to the main subject of this paper.

(2) When doing this type of experiment it is important to try to arrange the experimental conditions so that the proportion of fertilized eggs lies between about 0·3 and 0·7. If very high or very low proportions are obtained, discordant results may be encountered because of the large sampling errors that occur when the number of fertilized or unfertilized eggs in the suspension is small. For example in the first experiment in this series, which was rejected for this reason, there were 220 unfertilized and six fertilized eggs in the control suspension. This made α_N/α_C too high to be credible, because of the low value of α_C in the controls.

Table 1. *Proportion of fertilized* (f) *and unfertilized* (u) *eggs of* Paracentrotus lividus *in a suspension of homologous spermatozoa of known density* n

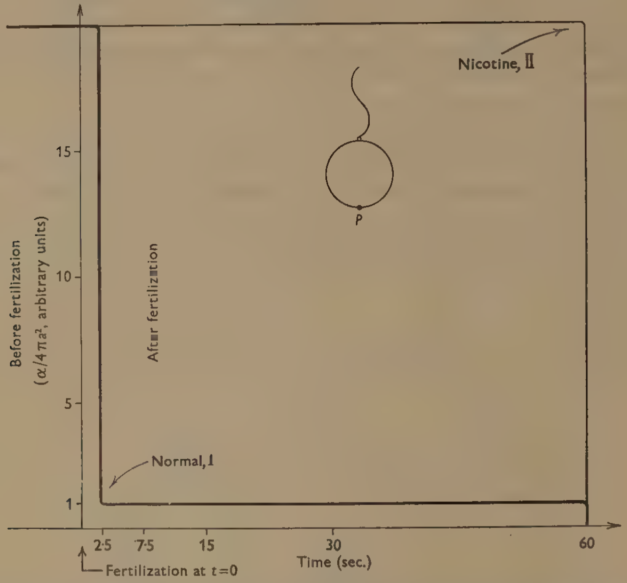
Sperm-egg interaction time, 25 sec. E, total number of eggs counted. N, eggs pre-treated for 2 min. with sea water containing nicotine (1/2000, v/v), C, controls, not pre-treated with nicotine. α is the slope of curve relating log u and t, in this instance 25 sec.

Exp. no.	n		f	u	E	α_N/α_C
1	$1{\cdot}06 \times 10^5$	N	0·274	0·726	299	1·2
		C	0·228	0·772	180	
2	$1{\cdot}24 \times 10^5$	N	0·275	0·722	176	1·7
		C	0·170	0·830	218	
3	$1{\cdot}88 \times 10^5$	N	0·239	0·761	431	1·8
		C	0·144	0·856	278	
4	$3{\cdot}14 \times 10^5$	N	0·573	0·427	206	9·6
		C	0·085	0·915	281	
5	$5{\cdot}24 \times 10^5$	N	0·737	0·263	224	4·0
		C	0·281	0·719	228	
6	$5{\cdot}25 \times 10^5$	N	0·765	0·235	217	5·3
		C	0·238	0·762	282	
7	$5{\cdot}57 \times 10^5$	N	0·840	0·160	263	4·3
		C	0·350	0·650	309	
8	$5{\cdot}57 \times 10^5$	N	0·790	0·210	248	2·2
		C	0·507	0·493	219	
9	$5{\cdot}63 \times 10^5$	N	0·425	0·575	214	2·6
		C	0·191	0·809	199	
10	$1{\cdot}68 \times 10^6$	N	0·667	0·333	219	2·5
		C	0·356	0·644	331	

(3) During these experiments it became clear that polyspermic eggs undergo their first cleavage markedly earlier than monospermic eggs. This phenomenon has interesting implications from the point of view of the mechanism of cleavage. As, however, it did not concern this investigation, the block to polyspermy in nicotine-treated eggs, the subject was not pursued.

(4) Reference has been made in this paper to unfertilized monospermic, polyspermic and activated eggs. All of these except the last have been repeatedly mentioned in previous papers. Pl. 5 *a* and *b* show how these different classes of eggs can be identified.

These experiments show without reasonable doubt that nicotine induces polyspermy by increasing the conduction time of the block to polyspermy and not by altering the egg surface in such a way that the probability of fertilization, i.e. α, is increased by the necessary amount. The probable forms of the block to polyspermy in nicotine-treated and untreated eggs are shown in Text-fig. 4.



Text-fig. 4. Curve I, variation in α per unit area ($\alpha/4\pi a^2$), at the point P on the egg surface, fertilization having occurred at the opposite pole at $t=0$. The first fall in α is due to the fast incomplete block to polyspermy reaching P. The second fall in α occurs when the complete block has covered the whole egg surface. Curve II, effect of nicotine on the block to polyspermy and therefore on α per unit area. The curves are, of course, idealized.

DISCUSSION

Time sequence of events. This is a convenient time to summarize our existing knowledge about the changes that occur in the sea-urchin egg immediately after fertilization. Contrary to earlier views, which in any case were not based on experimental evidence, the block to polyspermy is not an all-or-none process. After attachment of the fertilizing spermatozoon there passes over the egg surface a structural change which reduces the probability of re-fertilization by a factor of about 20. This change is completed in under 2 sec. At the end of it, however, the egg is not totally impermeable to supernumerary spermatozoa. Total impermeability is only achieved after some 60 sec., by which time the slow component of the block to polyspermy has completely covered the egg surface. The change in cortical structure which passes over the egg surface after fertilization, and whose conduction time is about 20 sec., is clearly a reflexion of this slow component, though the time relationships of the whole event suggest that the fertilization membrane, or the processes immediately connected with its elevation, may well play a role, though perhaps a relatively minor one, in the prevention of polyspermy.* These facts can be expressed in a different and more quantitative way; when eggs are mixed with spermatozoa at a density of 10^6/ml. the probability of a successful sperm-egg collision is about 0·05; that is to say, 1 in 20 collisions are, on the average, successful. After such a successful collision, the probability of a further successful collision, i.e. dispermy, falls to 1 in 400 in under 2 sec. At 60 sec. the probability of a successful collision falls to zero. Some confusion may have been caused by Just's remark (1939, p. 15) that 'in general, all normally monospermic eggs that I have studied are never polyspermic if they are in optimum condition'. This statement is of course incorrect. No mechanism could prevent polyspermy *ever* occurring because any change in egg structure must take a finite time to affect the whole of the egg surface. It therefore follows that under normal conditions of fertilization, polyspermy is not an impossibility but a rare event, the rarity depending on the speed at which the block to polyspermy covers the egg surface, that fraction of the total area of egg surface with which a spermatozoon can combine, the number of active spermatozoa in the neighbourhood of the egg in question, and the speeds at which the spermatozoa swim. When systematic counts are made of the incidence of polyspermy in egg suspensions which have been inseminated with spermatozoa at normal densities, the figures quoted above are found to be consistent with these counts. Naturally, the incidence of polyspermy goes down as the density of spermatozoa in contact with the eggs is reduced, for the simple reason that the lower the sperm density, the smaller the number of sperm-egg collisions that occur in a given time.

Polyspermy inducers. Treatments which cause polyspermy fall into two classes, over-insemination and chemical agents. Over-insemination is a method of forcing supernumerary spermatozoa into the egg both before the quick and slow blocks have covered the egg surface. The only chemical agent whose mode of action has been systematically investigated is nicotine, and the experiments described in this paper show that it induces polyspermy by increasing the conduction time of the block to polyspermy. The reasons for the view that this increase in conduction time is due to the abolition of the quick phase of the block have been given above. The question whether all chemical agents which induce polyspermy do so in this way cannot at present be answered. Some might well do so by the alternative method of increasing the probability of fertilization and therefore of re-fertilization. There is, at any rate, no structural similarity between several of the substances which induce polyspermy, though it is possible that the conduction mechanism is so delicately poised that interference with it can be achieved by non-specific means.

Mechanism of conduction. There has in the past been some discussion about the block to polyspermy being conducted through the egg cytoplasm or round the cortex. This question has become less important, if not academic, now it is known that the block to polyspermy has a fast component. Reference to the diffusion curves in the paper on this subject (Rothschild, 1949), apart from more general considerations, indicates that an intracellular diffusion mechanism is unlikely to account for this fast component. Recently, Runnström & Kriszat (1952) have published a paper in which they argue that the block to polyspermy is conducted round the cortex and not through the cytoplasm. This view is based on experiments involving the fertilization of damaged eggs. At fertilization, such eggs form unfertilized ex-ovates which can be fertilized later, as evidenced by the elevation of a fertilization membrane and a change in the consistency of the cytoplasm. These experiments probably confirm the view, expressed above, that the fast component of the block to polyspermy is a cortical reaction. As regards the slow component of the block, with which the visible change in the cortex is obviously connected, the position appears to the writer to be exactly as before, and unaffected by experiments on pathological eggs, which merely show that, as might be expected, maltreatment of an unfertilized egg damages the egg surface. There is evidence in this paper, e.g. pseudogamous fertilization, and in that of Runnström & Kriszat, to show that cytoplasmic changes occur after the attachment of the fertilizing spermatozoon to the egg surface. The time course of these changes is consistent with the hypothesis that the complete block to polyspermy is achieved by intracellular diffusion.

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* The conduction times of the cortical change and of the wave of exploding cortical granules described by Endo (1952) are sufficiently similar to make it very probable that these two phenomena are different aspects of the same process.

SUMMARY

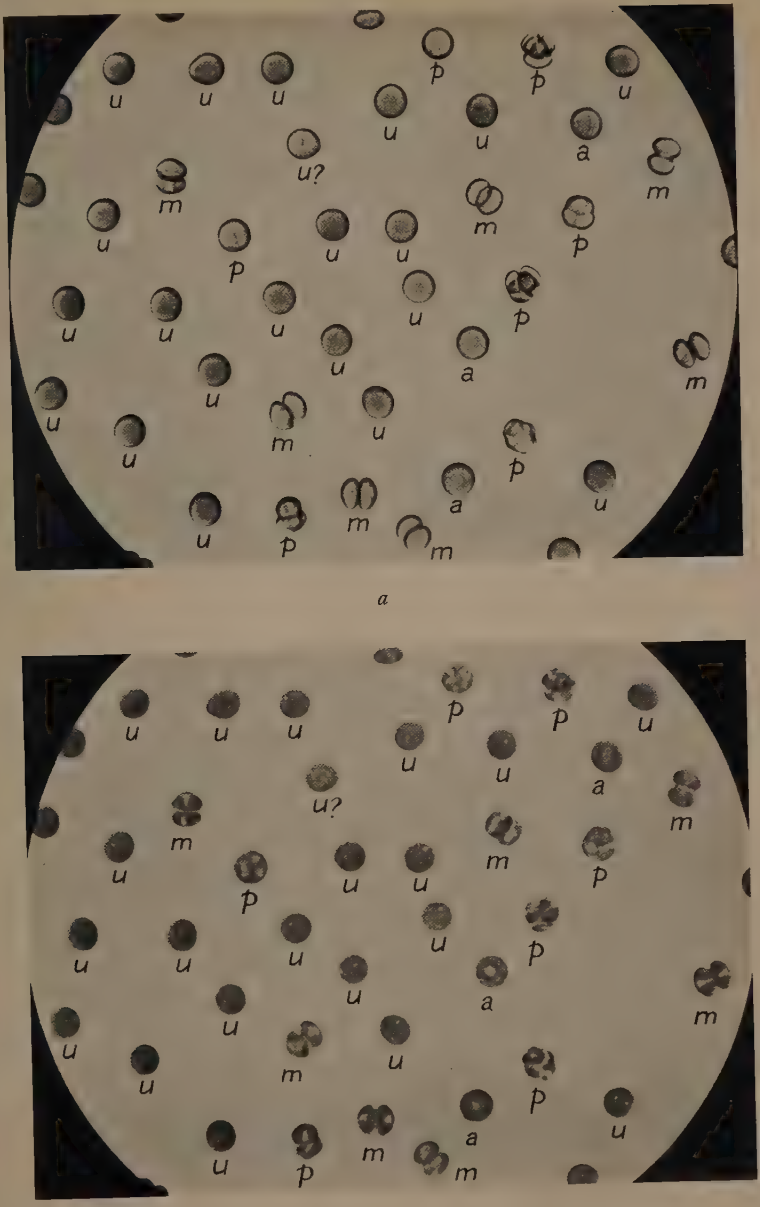
1. Treatment of unfertilized sea-urchin eggs with sea water containing nicotine is known to induce polyspermy when the eggs are subsequently inseminated with homologous spermatozoa, at densities which cause a very small amount of polyspermy in untreated eggs.

2. If nicotine were to increase the speeds at which sea-urchin spermatozoa swim, the chances of fertilization, and therefore of polyspermy, might be increased. Nicotine does not increase sperm speeds; in addition, it causes a sharp reduction in the O_2 uptake of these spermatozoa.

3. The only other ways in which nicotine could induce polyspermy are by altering the egg surface in such a way that the probability of fertilization is increased by a factor of about twenty; or by lengthening the conduction time of the block to polyspermy. Experiments described in this paper show that the first explanation is untenable and therefore that the second is the correct one. It is concluded that nicotine abolishes the fast incomplete block to polyspermy and that over-exposure to this substance probably abolishes the block to polyspermy altogether.

4. Polyspermic eggs divide sooner than monospermic ones.

5. When, as in these experiments, eggs are allowed to interact with spermatozoa for known times, and then functionally separated by immersion in hypotonic sea water, some eggs, presumably those which sustain a successful sperm-egg collision at the end of the interaction time, are activated but not fertilized by the spermatozoon,



ROTHSCHILD—THE FERTILIZATION REACTION IN THE SEA-URCHIN

as in the pseudogamous nematodes. Cleavage does not occur though the egg nucleus swells.

6. Previous results in the same field and observations by other workers are discussed.

I am much indebted to the Director and Staff of the Zoological Station, Naples, for their kindness and hospitality, and to the Medical Research Council for the provision of a laboratory assistant.

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EXPLANATION OF PLATE 5

(*a*) Suspension of eggs of *Paracentrotus lividus*, with jelly, showing *u*, unfertilized eggs; *m*, monospermic eggs; *p*, polyspermic eggs; *a*, activated eggs.

(*b*) The same suspension deliberately photographed out of focus, to show *u*, eccentrically placed nuclei in unfertilized eggs; *p*, asters in polyspermic eggs which have failed to divide; *a*, central and enlarged female nuclei in activated eggs.

Note doubtful unfertilized egg, *u*? Magnification, 54.

THE RELEASE OF PHOSPHATE FROM THE ELECTRIC ORGAN OF *TORPEDO*

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INTRODUCTION

The electroplates of the electric organ of *Torpedo* are embryonically derived from striated muscle fibres which lose their ability to contract and develop greatly expanded end-plate surfaces (cf. Rosenberg, 1928). From consideration of the thermodynamics of the *Torpedo* organ discharge, Bernstein (1912) concluded that the electric energy of the discharge was derived from a difference between the concentration of ions on the inside and outside of the electroplate, and was released by a breakdown in the selective ion permeability of the innervated surface of the electroplate. One ion species whose movement would be expected to play a part in generating the electric discharge is the sodium outside the electroplate. If, however, the activation of the electric organ is at all analogous to that of the motor end-plate, it is necessary to invoke the movement of additional ions (Fatt, 1950; Fatt & Katz, 1951). Among the ions in the electric organs which could contribute to the discharge there is the internal phosphate of the electroplate (Kisch, 1930; Baldwin, 1933). In the present experiments analyses have been made on the fluid issuing from a perfused electric organ during stimulation to find out if an outward movement of phosphate takes place during activity. The movement of other anions has also been studied.

The embryonic origin of the *Torpedo* organ and the results of Feldberg & Fessard (1942) on the effect of injected acetylcholine strongly suggest that the end-plate and electroplate are similarly activated. There is, however, some difference between the two; for Fessard & Tauc (1951) have reported that the resting potential of the electroplate is about one-half the size of its action potential (the latter calculated from the discharge of the whole organ). Such a result would not be expected if the discharge were effected by a non-specific increase in membrane permeability, as apparently occurs during the activity of the motor end-plate (Fatt & Katz, 1951). The electric response is in this respect similar to that found in *Electrophorus* (Keynes & Martins-Ferreira, 1952). The latter tissue, however, possesses the properties of direct excitation and conduction which may have been lost in *Torpedo*. As a part of the series of experiments described in this paper the effect of perfusing an organ with acetylcholine has been studied to extend the earlier observations of Feldberg & Fessard (1942).

METHODS

Dissection and perfusion technique

Large fresh specimens of the electric ray, *Torpedo marmorata*, were used. The dissection was carried out from the ventral surface. The second, third and fourth nerves to the electric organ and their accompanying arteries were dissected and tied off before the organ was removed from the body.

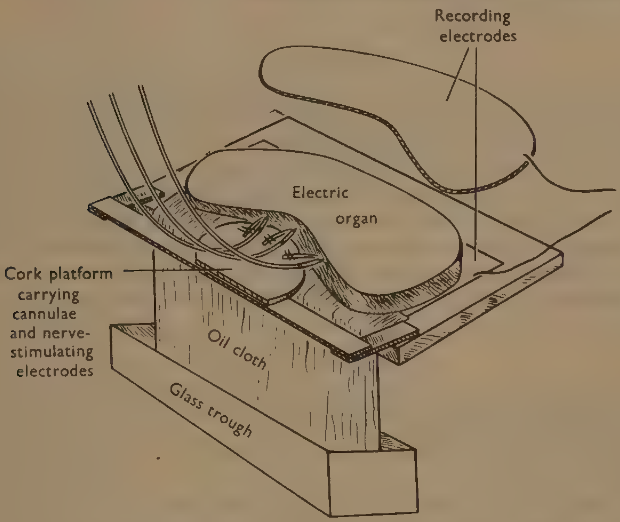
Recording electrodes
Electric organ
Cork platform carrying cannulae and nerve-stimulating electrodes
Oil cloth
Glass trough

Fig. 1. Arrangement of electric organ for perfusing, stimulating and recording.

The isolated organ was placed on a cork support in which was embedded a recording electrode and over the edge of which hung an oil cloth apron. This apron guided the venous outflow into a glass basin placed beneath the organ. The support also held a small cork platform to which nerve-stimulating electrodes were affixed. The platform fitted against the organ below the level of emergence of the nerves. The arteries were cannulated and the cannulae attached firmly to the cork platform. The nerves were then placed over the stimulating electrodes and a large flat recording electrode was placed over the whole organ. The complete assembly is shown in Fig. 1.

To insure a uniform rate of perfusion each artery was connected to a separate dropper and adjusting clip. The total rate of flow through the arteries was 4–8 ml./min. The perfusion fluid had the composition (in terms of mmol./kg.

water): sodium 275 mM., potassium 7·6 mM., calcium 4·3 mM., magnesium 1·4 mM., urea 415 mM., bicarbonate 1·1 mM., chloride 294 mM. In some experiments prostigmine bromide was added in a concentration of 5–20 μM. When the amino-acid composition of the effluent was investigated the urea was omitted without any substitution being made for it.

The organ could be perfused with solutions containing acetylcholine (ACh) or choline chloride. These were admitted to the organ by changing the reservoir supplying the perfusion fluid.

The experiments were done at a temperature of about 23° C. At the end of each experiment Evans blue was injected into the arterial system and from its distribution the efficiency of the perfusion was judged.

Electrical recording

The recording electrodes were made of large silver sheets whose surfaces were amalgamated with mercury and electrolytically coated with chloride. They were separated from the organ by pads of cotton gauze impregnated with agar made up in perfusion fluid. Although the electrodes had to be rechlorided frequently they had the advantage of a very low resistance (*c.* 3 Ω) which allowed the maximum current of the electric organ to be recorded. The response of the organ was determined as the current flowing through a small resistance. The current was measured either by monitoring with an oscillograph the potential difference developed across 1–3 Ω or, for long lasting changes, by passing the current through a low-resistance milliameter. The resistance of the preparation was also measured.

Analysis of perfusate

As a preliminary to any analysis the samples of venous effluent were centrifuged and the sediment, consisting of blood corpuscles, discarded.

The total and inorganic phosphorus was determined by the method of Berenblum & Chain (1938).

Sulphate was determined gravimetrically after precipitation with $BaCl_2$.

Paper chromatography was used for analysis of phosphate esters, non-volatile organic acids and reducing substances. Taking 25 ml. of each sample, salts and urea were removed (after precipitation of sulphate) by use of an acid-treated cation exchange resin (Zeo-Karb 215), and the solutions concentrated *in vacuo* over NaOH. The solvents used for irrigating the papers were *n*-butanol-acetic acid-water (Partridge, 1948), picric acid-*t*-butanol and isopropyl ether-formic acid (Hanes & Isherwood, 1949). The papers were developed with the molybdate-perchloric acid reagent for phosphates (Hanes & Isherwood, 1949), and $AgNO_3$-NH_3 for reducing substances (Partridge, 1948); the papers treated with $AgNO_3$ were examined in ultra-violet light to disclose fluorescent silver salts of organic acids.

In two cases where amino-acids were under investigation, 30 ml. samples of the centrifuged, urea-free effluent were passed through the ion exchange resin; the column was washed free from chloride and eluted with 0·5 N-NH_4OH; and the eluate was dried *in vacuo* over H_2SO_4. The chromatograms were irrigated with phenol-NH_3-coal gas after which areas corresponding to aspartic acid, glutamic acid, glycine and alanine were cut out and assayed colorimetrically (Fowden, 1951).

For analysis of choline esters the perfusate was brought to pH 4 and dried *in vacuo*. The residue was extracted with absolute alcohol and the alcohol solution evaporated *in vacuo*.

The solid so obtained was extracted with acetone and the supernatant phase collected, put on paper, and irrigated with *n*-butanol. After irrigation and drying at room temperature the chromatogram was cut in sections and the ACh activity determined by assay on leech muscle.

RESULTS

Perfusion of the electric organ

Auger & Fessard (1941) found that bathing fragments of *Torpedo* organ in solutions of drugs which modify the response at the neuromuscular junction was not effective until incisions had been made into the columns of electroplates. This indicated that diffusion of substances through this tissue is very slow. Consequently, it seemed necessary to use the perfusion method of Feldberg & Fessard (1942) to investigate the liberation of substances by the electroplates.

The electric organ of *Torpedo* is supplied by four nerves each of which, in a fully grown specimen of the species used here, has a diameter of about 4 mm. An artery accompanies each nerve. The artery is very much smaller than the nerve, having a diameter of about 0·5 mm. Near their entry into the electric organ the arteries lie in the connective tissue wrapped around the nerves and cannot be readily found unless filled with blood. The artery accompanying the first, i.e. the most anterior, nerve was not used since it is smaller than the others, is frequently divided before entering the organ, and supplies only a small area.

In the present experiments it was difficult to achieve a uniform perfusion of the whole organ. The injection of Evans blue frequently revealed that large areas, particularly in the more peripheral parts were not being reached. Before collecting samples of effluent or adding ACh to the ingoing fluid the perfusion was allowed to proceed for at least 30 min. Most of the blood contained in the organ was washed out during the first 2–3 min. of perfusion. After this there was a steady thin stream of blood corpuscles in the venous fluid coming from less effectively perfused regions. The electric response after perfusing for 30 min. was about twice as large as it was immediately after dissection. This increase was probably the result of reversing the unfavourable conditions which had arisen during the course of the 1½–2 hr. of dissection, while the organ was in the anoxic fish.

In all cases in which ACh was added to the perfusion fluid there was a marked reduction in the rate of flow. With a constant perfusion pressure the rate of flow fell usually to one-half. This suggests that ACh has a vaso-constrictor action in the electric organ. Alternatively, though this is not very likely, there might have been contraction in the muscles on the edge of the organ causing a folding of the arteries. With the rate of perfusion used, if immediate mixing of all the fluid in the organ occurred, about 30 min. would be required to exchange two-thirds of the fluid in the organ (organ *c.* 180 cm.^{3}, perfusion rate *c.* 6 cm.^{3}/min.); actually it was observed that only about 10 ml. of dye solution had to be perfused into an organ for the venous effluent to appear as deeply coloured as the ingoing fluid. The rate of exchange therefore depends mainly upon the rate at which substances can diffuse between the bulk of the tissue and the blood vessels.

Liberation of anions

The main object of the present research was to investigate the liberation of anions from the electroplate during activity. The organ was brought into activity by stimulating the three nerves at a rate of 10 per sec. The venous effluent was collected and grouped into a series of samples covering a period of about 30 min. before and after the onset of stimulation. Chemical analyses were performed on these samples.

The amplitude and duration of the individual discharge and its variation during the course of stimulation was roughly measured in order to estimate the quantity of charge passed by the organ when stimulated to exhaustion. The response to individual stimuli was seen to fall to one-third or less after 1000 shocks. In some experiments stimulation was discontinued after 5 min., at which time the response was down to about 1% of its initial value; in other experiments stimulation was continued as long as samples were collected. This variation in procedure did not affect the results. In experiments in which chemical analyses of the perfusate were performed the total charge passed by the electric organs amounted to about 0·5 coulomb. The samples from a few organs in which the total charge was considerably below this quantity were discarded.

The anions for which quantitative analyses were performed were phosphate, sulphate, asparate and glutamate. Under the conditions of the estimation (Berenblum & Chain, 1938) all of the phosphorus in the effluent reacted as orthophosphate. Typical values for the concentrations of the ions in the effluent from an organ before stimulation (perfusion rate 5 ml./min.) were:

Phosphate	0·1 μmol./ml.	Glutamate	0·1 μmol./ml.
Sulphate	0·3 μmol./ml.	Aspartate	0·02 μmol./ml.

(The absolute values for glutamate and aspartate were accurate only within a factor of two, although their ratio (5 : 1) was more precise.)

On the start of stimulation the phosphate content of the fluid issuing from the organ rose appreciably above the resting level. There was a lag of about 5 min. before a maximum was reached. The rising phase could not, however, be accurately determined, since the time covered by each sample was relatively long. After reaching a maximum the phosphate concentration fell at such a rate that one-third of the extra phosphate concentration remained after 30 min. A typical experiment is illustrated in Fig. 2. In four other experiments the peak increase in phosphate concentration on stimulation was 95, 85, 63 and 65% of the resting value.

It should be noted that, while a large electric response was elicited only during the first 2 min. of stimulation, the phosphate release followed a much more prolonged time course. If the phosphate is liberated from the electroplate as an immediate accompaniment of the electric response, then the time course of the observed release from the whole organ is determined almost entirely by the time required for the phosphate to be removed from the neighbourhood of the electroplate. In order to arrive at an estimate of the additional phosphate released as a result of stimulation the phosphate curves were extrapolated assuming that the decay from the peak to the resting level was exponential with a fall to $1/e$ in 30 min. The average extra phosphate release thus obtained was about 10 μmol. for stimulation to exhaustion, and the charge carried by the ions corresponded to about 20 μequiv.

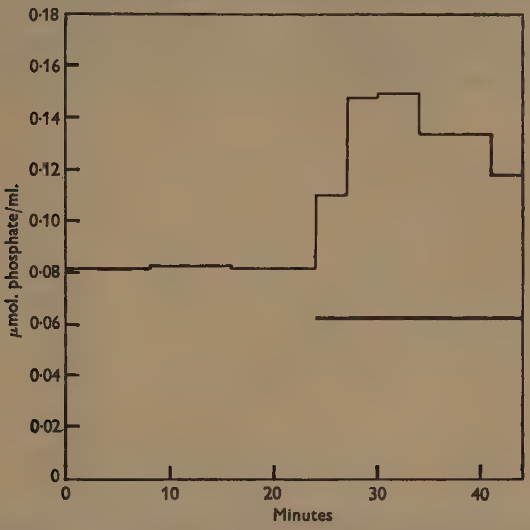

Fig. 2. Phosphate concentration in the effluent from an electric organ. The organ was stimulated during the period indicated by the heavy line. Rate of perfusion was 5·5 ml./min.

An attempt was made to follow the phosphate release from organs when ACh was added to the perfusion fluid as in the experiments described in the following section; however, no consistent change in the phosphate concentration of the effluent could be detected.

Stimulation to exhaustion did not change the sulphate concentration in the venous effluent. As regards amino-acids, only the ratio of glutamate and aspartate to glycine and alanine could be satisfactorily determined because of the possibility of the loss of some fraction of the samples in the rather involved analysis. In the two experiments in which amino-acids were assayed in this way the ratio did not change on stimulation. Further analysis of the effluent did not lead to the detection of any other organic anions (see p. 70 for methods used in this connexion).

Acetylcholine as chemical transmitter

Since the functional analogy of the *Torpedo* electric organ with the motor end-plate depends on the role of ACh as a chemical transmitter it was considered important to extend the work of previous investigators on the effect of injected ACh. Feldberg & Fessard (1942) found that the open-circuit response to ACh under the most favourable conditions had a maximum of the order of 20 mV.

In the present experiments ACh was run into the organ by steady perfusion with a solution of constant ACh concentration rather than by the rapid injection technique employed by Feldberg & Fessard. Under such conditions ACh concentrations of 0·1–1·0 mM. were required to give response of the order of 0·1–1·0 mA. into a low external resistance. Considering that the resistance of the organ was about 60Ω these responses correspond to open circuit potential differences of 6–60 mV. (See below for the assumption involved in this calculation.) Further increase in ACh concentration did not give a larger response. Previous perfusion with prostigmine in a concentration of 10–20 μM. increased the response and reduced the amount of ACh necessary within the stated ranges of current and concentration, respectively. The response to ACh obtained from an organ which had been perfused with prostigmine solution is illustrated in Fig. 3. This response is typical of that obtained when the perfusion progressed reasonably well. Even in this experiment, however, the rate of perfusion fell from 4·7 to 3·8 ml./min. immediately on admitting ACh to the organ. The peak current was 0·57 mA. and total charge passed, obtained from the area of the current-time curve, was 0·103 coulomb. Taking the source of the e.m.f. to be in series with the resistance of the organ (58Ω) and the recording system (7Ω), the open circuit potential difference can be obtained by multiplying the ordinates of Fig. 3 by (58+7)Ω (cf. Albe-Fessard, 1950). The peak value of the open circuit potential difference is thus 37 mV.

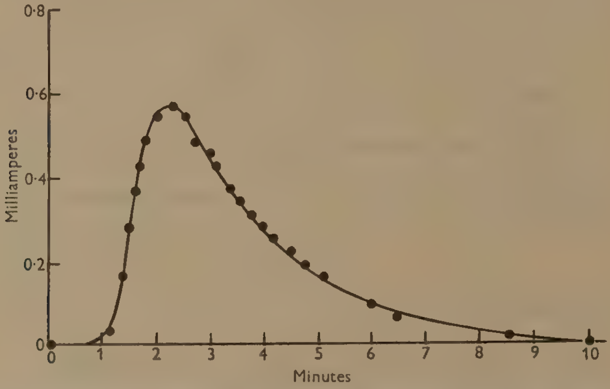

Fig. 3. Response of an electric organ to perfusion with 0·50 mM. ACh. The organ had been perfused for the previous 3 hr. with a solution containing 10 μM. prostigmine. The initial rate of perfusion was 4·7 ml./min.; after admitting ACh it fell to 3·8 ml./min. About 1 min. was required to change the perfusion fluid in the line leading up to the organ.

The rapid fall of the response is probably due to an exhaustion of the ACh-receptor similar to that observed in muscle when using high concentrations of ACh (Fatt, 1950). A response could be elicited by perfusing with choline. This response, which would not be affected by cholinesterase, showed a somewhat less precipit rise and fall, but even with very high concentrations of choline (choline chloride replacing part of the NaCl in the perfusion fluid) the peak current was no greater than with ACh.

To test the specificity of the electroplate reaction for ACh the relative effectiveness of choline and ACh were compared on one organ. The experiment is illustrated in Fig. 4 where the magnitude of the peak current is shown for each test solution. Solutions of the compositions indicated were perfused into the organ until a maximum response was reached. The organ was then washed by perfusing with normal (prostigmine) solution and, when the base-line was regained, the next test solution was run in. Although the organ had been perfused with prostigmine for 1½ hr. before the first test with ACh, there was still a large increase in the ACh-response during the course of the 2 hr. illustrated. This must be ascribed to continued inactivation of cholinesterase. This experiment shows that a choline concentration more than 100 times that of ACh is required to produce the same response.

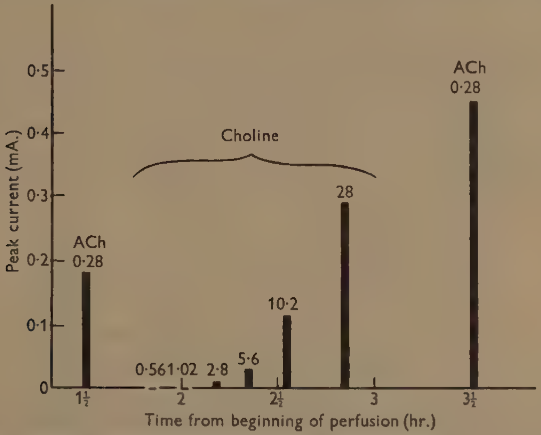

Fig. 4. Peak current of the response of an electric organ to ACh and choline. Concentrations in mM. are indicated. The perfusion fluid contained 10 μM. prostigmine. Rate of perfusion was about 6 ml./min.

An analysis of the effluent from a stimulated, prostigminized organ for choline esters revealed the presence of ACh but no other active substances. Nevertheless, in view of the lability of these substances and the unsatisfactory techniques employed to prepare the venous fluid for chromatography the presence of other choline esters cannot be completely excluded. If, however, such esters are no more labile than ACh, then they represent less than one-tenth of the total activity of the effluent.

DISCUSSION

A release of anions from the electric organ is of interest because the charge which is thus carried across the electroplate membrane may play an important part in generating the electric discharge. An estimate can be made of the total quantity of ions which must cross the electroplate surfaces in order to produce the discharge of the whole organ. The organ is made up of a number of prisms in parallel, in each of which the electroplates are arranged in series. During the discharge each of the electroplates adds to the total e.m.f. and boosts the current by allowing ions to move down an electrochemical potential difference across its active membrane. (This assumes that the active-membrane potential of the electroplates is independent of the current drawn, a condition which is in practice probably fulfilled; cf. Albe-Fessard, 1950). The number of equivalents of ions which must therefore move across all the active electroplate membranes is the product of the charge delivered by the organ and the number of electroplates in series, all divided by the Faraday. For the fish used in these experiments the number of cells in each prism is about 400 (Rosenberg, 1928). Hence, for a discharge of 0·5 coulomb the ion transfer across all the active cell surfaces is $0{\cdot}5 \times 400/10^5 = 2 \times 10^{-3}$ equiv.

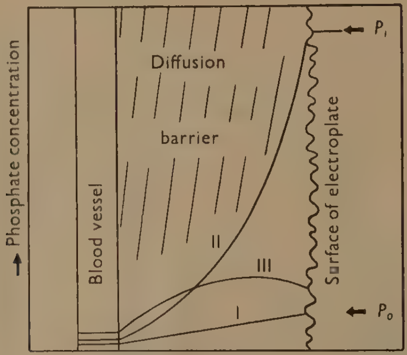

Fig. 5. Sketch to explain the exchange of phosphate between the electroplate and blood vessel; I, electroplate at rest; II, during activity; III, immediately following activity. P_o/P_i represents the external-internal concentration ratio maintained by the electroplate at rest.

The amount of phosphate which has been recovered in these experiments is sufficient to account for only 1 % of this transfer of charge. It might be argued that a much greater quantity of phosphate is released from the electroplates than has been recovered from the venous effluent. If one could assume the existence of an appreciable diffusion barrier between the electroplate and blood vessel the scheme illustrated in Fig. 5 can be envisaged. At rest there is a constant phosphate gradient from the surface of the electroplate to the blood vessel (1). During the brief period of activity the concentration in the immediate neighbourhood of the electroplate rises to nearly that of the interior (III), but because of the diffusion barrier the phosphate does not move far and is taken up again by the electroplate during recovery (III).

As an alternative to an increase in membrane permeability the observed phosphate release might result from an increase in the concentration of intracellular inorganic phosphate. In a fresh organ the splitting of high energy phosphate ester is presumed to be coupled with activity so as to restore the balance of ions existing before the discharge. The experiments of Kisch (1930) and Baldwin (1933), however, suggest that an insufficient amount of phosphate esters would have been present in the organs used in the present experiments to account for the large *relative* increase in phosphate liberation.

The small size of the response obtained with ACh compared with that from nerve stimulation and the enormous concentrations required to elicit it is another case of the small effects obtained from perfusion of the tissue. An explanation can again be given in the defects of the perfusion technique. It may be, however, that there are other reasons which follow from the structural organization of the electroplate. For instance, it is possible, as suggested to us by Prof. Fessard, that the non-innervated surface of the electroplate is affected by ACh to some extent as well as the innervated one. An important point to consider here is the chemical specificity of the electroplate reaction for ACh. In this respect the results strengthen the belief that ACh serves the same role here as at the motor end-plate.

SUMMARY

1. A technique is described for perfusing the isolated electric organ of *Torpedo*. With a constant perfusion pressure the admission of acetylcholine is found to cause an appreciable reduction in the rate of flow.

2. Orthophosphate, sulphate, glutamate and aspartate have been identified as anions which pass from the electric organ into the fluid perfusing through it.

3. With a perfusion rate of 4–8 ml./min., repetitive stimulation of the nerve caused an increase in the rate of phosphate released from the organ. The phosphate concentration in the venous effluent rose to a maximum of about 180% of the resting level, and stimulation to exhaustion resulted in the release of an additional 10 μmol. of phosphate.

4. Perfusion of an electric organ by acetylcholine elicited a slowly developing response amounting at the most to about 1 mA. when passed into a low resistance and lasting for about 7 min.

5. The possible role of intra-electroplate anions in generating the discharge of the *Torpedo* electric organ is discussed. The additional phosphate released from the organ on stimulation accounts for only 1% of the charge which crosses the active electroplate membrane.

We wish to express our thanks to Prof. A. Fessard for his encouragement and advice at every stage of the work. We are indebted also to Prof. R. Weill and other members of the Institut de Biologie Marine for the facilities of the laboratory.

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EXCHANGES OF SODIUM AND POTASSIUM IN MOSQUITO LARVAE

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INTRODUCTION

In an earlier paper (Ramsay, 1950) an account was given of osmotic regulation in mosquito larvae of the genus *Aedes*. In the case of the fresh-water species *A. aegypti* L. with which the present paper is concerned, the situation is as follows. The surface of the body is relatively impermeable to water except over the anal gills (Wigglesworth, 1933*a*). Water enters the body through these organs, which are also capable of secreting chloride into the haemolymph against a concentration gradient (Koch, 1938). A fluid, very slightly hypotonic to the haemolymph, is elaborated by the Malpighian tubules. Some of this fluid appears to pass forwards through the mid-gut, eventually to be reabsorbed into the haemolymph through the caeca; some of it passes down the intestine to the rectum where it becomes hypotonic to the haemolymph before being eliminated from the body.

In a subsequent paper (Ramsay, 1951) the part played by the Malpighian tubules was further studied, particularly in relation to the excretion of sodium. It was found that when the larvae were kept in tap water the concentration of sodium in the fluid from the Malpighian tubules was always less than that in the haemolymph, and that when the larvae were kept in various concentrations of NaCl (up to 1% NaCl, which was the maximum tolerated) the concentration of sodium in the tubule fluid, although increased, never exceeded that in the haemolymph. It thus appeared that when the external medium was poor in salts the Malpighian tubules could contribute to the work of salt retention by excreting a fluid containing less sodium than the haemolymph; but no evidence was found to suggest that they could excrete a fluid containing more sodium than the haemolymph under conditions in which sodium was present in excess.

At that time sodium was the only element whose concentration could be determined in the small volumes of fluid available. The technique of flame photometry has now been extended to make possible the simultaneous determination of sodium and potassium in samples of the order of 10^{-3} mm.^{3} in volume (Ramsay, Brown & Falloon, 1953). With this method the study of salt and water balance in mosquito larvae can be carried a stage further.

MATERIAL AND METHODS

Larvae of *A. aegypti* were reared to the fourth instar as described in the earlier papers referred to above. They were then removed to clean tap water and kept in this medium for at least a week before being subjected to the various treatments which will be described in due course.

The methods used for collecting various fluids were substantially the same as already described in the earlier papers, but certain modifications were found to give better results.

The collection of representative samples of rectal fluid has always presented difficulties. It is possible to insert a pipette into the rectum via the anus and to collect the fluid which happens to be present at the time, but the amount thus available is usually inconveniently small. It is also possible to tie a cannula into the anal canal and to collect over a period, but this has the disadvantage that the fluid passes into the cannula almost as soon as it reaches the rectum from the intestine, and does not remain in contact with the rectal epithelium as long as it does in the normal animal. In the present work the rectal fluid was collected as it was voided through the anus. The larva was first washed quickly with distilled water and then carefully dried on cigarette paper; it was secured in the usual way by a snare around the respiratory siphon and by a ligature drawn tight around the neck, and was thus held down upon a hollow-ground glass slide. A small quantity of liquid paraffin was added, sufficient to cover the larva except for the opening of the respiratory siphon. A fine capillary pipette was then applied to the region of the anus, and any fluid emerging rose at once into the pipette.

For the collection of intestinal fluid the larva was secured as above and was immersed in the medium in which it had been kept prior to the experiment.

To collect midgut fluid a cannula was inserted through the mouth to about half way down the midgut. During collection the larva was kept in moist air, impaled upon the cannula. The insertion of the cannula is not difficult but the food and faeces in the gut often block it, so that the proportion of failures in this operation is relatively high.

Four determinations (of sodium and potassium simultaneously) were made on each sample and were compared with at least four determinations on a reference solution, the mean values being used for calculation. The standard error of figures given in the tables is believed to be of the order of ±2% of the concentrations. The variation between samples of the same fluid taken from different animals was of a much greater order (standard deviation about ±10% or greater) so that the errors of analysis do not enter significantly into any of the conclusions which are drawn. It is one of the shortcomings of the method that when the concentration of sodium exceeds that of potassium by a factor of 20 or more it is not possible to maintain the potential accuracy for both elements. This situation arises in the case of the haemolymph, and where relatively small differences in concentration were under study the two elements were determined separately (Tables 1 and 2).

Concentrations are expressed in mM./l. or m.equiv./l. Since in earlier work concentrations were expressed as % NaCl it may be helpful to state the relation 1% NaCl ≡ 171 mM./l. NaCl.

Where any doubt appeared to exist about the reality of differences between one fluid and another the '*t*' test for significance was applied, and the values of *P* were obtained from the tables of Fisher & Yates (1938). The usual conventions are adopted, i.e. $P < 0.05$ is significant, $P < 0.01$ is highly significant.

RESULTS

(1) *Sodium and potassium content of whole larvae.* Fifty larvae were taken from the stock in tap water, washed and dried with cigarette paper and then weighed in a platinum crucible. They were then dried and ashed at 600° C. and the ash was extracted with dilute HCl. Determination of sodium and potassium in this extract showed that these elements were present in whole larvae in the following amounts:

Sodium	0·066 m.equiv. per gram wet weight.
Potassium	0·033 m.equiv. per gram wet weight.

In a later experiment larvae from another culture were used. They were first placed for 5 days in a slow current of distilled water and then divided into two lots, one being placed in NaCl, 17 mM./l., and the other in KCl, 17 mM./l. After 5 days in these solutions fifty larvae were taken from each, washed quickly in distilled water, dried and analysed as above. The results are given below:

	Medium, NaCl, 17 mM./l. (m.equiv./g.)	Medium, KCl, 17 mM./l. (m.equiv./g.)
Sodium	0·080	0·074
Potassium	0·027	0·038

These changes in the sodium and potassium content of the larvae very clearly reflect the changes in the external medium, though in very much less degree. This is as one might expect, and by themselves these figures tell us very little. When they are considered in relation to similar figures for the haemolymph further conclusions can be drawn.

(2) *Sodium and potassium concentrations in the haemolymph.* Larvae were taken from stock and placed in a slow current of distilled water for 7 days. At the end of this time haemolymph was collected from six larvae and the remainder were divided between the following four solutions: NaCl, 85 and 1·7 mM./l.; KCl, 85 and 1·7 mM./l. After 5 days in these solutions six larvae were taken from each and the haemolymph collected. The results of the analysis are presented in Table 1.

Table 1. *Effects of different external media upon the composition of the haemolymph*

(Concentrations in m.equiv./l.)

Distilled water			NaCl, 85 mM./l.			NaCl, 1·7 mM./l.			KCl, 85 mM./l.			KCl, 1·7 mM./l.		
rial	Na	K	Serial	Na	K	Serial	Na	K	Serial	Na	K	Serial	Na	K
1	89	4·1	7	120	—	13	96	—	19	—	4·4	25	—	4·4
2	89	3·1	8	115	—	14	90	—	20	—	5·3	26	—	3·9
3	75	3·0	9	112	—	15	107	—	21	—	6·9	27	—	4·1
4	91	3·5	10	100	—	16	103	—	22	—	5·0	28	—	4·6
5	85	2·4	11	115	—	17	100	—	23	—	5·9	29	—	4·1
6	93	2·7	12	119	—	18	106	—	24	—	6·5	30	—	4·0
v.	87	3·1	—	113	—	—	100	—	—	—	5·7	—	—	4·2

The figures in Table 1 show that the sodium and potassium concentrations in the haemolymph remain relatively constant in the face of considerable changes in the external medium. At the same time the small differences observed, e.g. in the sodium concentrations 113 – 100 and 100 – 87 m.equiv./l., are real ($P < 0{\cdot}01$), and therefore the internal medium does in fact follow the changes in the external medium, albeit to a limited extent.

Since the 'whole' larvae considered in the previous subsection came from a different culture and were not exposed to the same solutions as the 'haemolymph' larvae now under discussion, some caution is necessary when comparing the figures. Nevertheless, it seems clear that the sodium:potassium ratio in the whole larva is about 2·5:1, whereas in the haemolymph it is about 25:1. If we assume that the haemolymph is some 50% of the wet weight the change in the sodium content of the whole larvae (as between different external media) is very nearly accounted for by the change in the concentration of sodium in the haemolymph. Presumably most of the sodium is in the haemolymph and not in the tissues. But the change in the potassium content of the whole larva is very much greater than can be accounted for by the change in the haemolymph. Presumably most of the potassium is held in the tissues, and furthermore the amount so held is dependent upon the availability of potassium in the external medium. At first sight it is surprising, in view of the very low concentration of potassium in the haemolymph, that these larvae can survive some 3 weeks or longer in a current of distilled water without losing all their potassium; but it seems likely that the tissues constitute a reserve from which the low potassium concentration of the haemolymph can be maintained for a long time.

(3) *Uptake by the anal gills.* In the experiment described in § (2) above, all the larvae were exposed to distilled water before being placed in salt solutions; the figures in Table 1 can therefore be used to show that the larvae are able to take up sodium and potassium from the external medium even when the concentrations of these elements in the external medium are lower than in the haemolymph. The demonstration by Koch (1938) that chloride is taken up from the medium by the anal gills and secreted into the haemolymph against the concentration gradient makes it seem highly probable that these same organs are also concerned in the active uptake of cations.

As Wigglesworth (1933*a*) has shown, it is possible to destroy the anal gills by exposing the larvae to 5% NaCl for 2–3 min. Gill-less larvae were prepared in this way and, together with an equal number of normal larvae from the same culture, were placed in a slow current of distilled water. After 5 days some (both gill-less and normal) were transferred to KCl, 1·7 mM./l.; after 8 days others were transferred to NaCl, 85 mM./l. Haemolymph was collected after 12–14 hr. exposure to these solutions. This short exposure was suggested by the work of Wigglesworth (1938), who found that after transference from distilled water to salt solutions the larvae showed an initial period of over-compensation during which abnormally high values of chloride concentration were found in the haemolymph; it was hoped that over-compensation would exaggerate any differences which might exist between gill-less larvae and normal larvae.

The results of these experiments are summarized in Table 2, and the differences as between gill-less and normal larvae are highly significant. They support the view that the anal gills are able to take up sodium and potassium from the external medium and secrete them into the haemolymph against the concentration gradient.

Table 2. *Effects of removal of the anal gills upon the uptake of sodium and potassium from dilute external media*

(Concentrations of sodium and potassium in the haemolymph, m.equiv./l.)

Distilled water→NaCl 85 mM./l.				Distilled water→KCl 1·7 mM./l.			
Normal		Without gills		Normal		Without gills	
Serial	Na	Serial	Na	Serial	K	Serial	K
31	131	37	85	43	7·8	49	4·2
32	129	38	112	44	5·6	50	4·7
33	103	39	117	45	8·0	51	5·6
34	116	40	99	46	6·2	52	3·4
35	129	41	90	47	5·3	53	4·1
36	110	42	78	48	5·7	54	3·8
Av.	120	—	97	—	6·4	—	4·3

(4) *Excretion by the Malpighian tubules.* Reasons have been given (Ramsay, 1951) for believing that the fluid which passes down the intestine is mainly derived from the Malpighian tubules. Collections have been made of this fluid from larvae kept in a current of distilled water (5 days): in NaCl, 85 mM./l. (10 days); and in KCl, 85 mM./l. (8 days). All these larvae were taken from the same culture. The results of the analysis are presented in Table 3.

Table 3. *Effects of different external media upon the composition of the intestinal fluid*

(Concentrations in m.equiv./l.)

Distilled water			NaCl, 85 mM./l.			KCl, 85 mM./l.		
Serial	Na	K	Serial	Na	K	Serial	Na	K
55	23	81	63	101	73	67	31	128
56	26	87	64	72	92	68	14	140
57	18	106	65	66	94	69	26	151
58	16	104	66	45	101	70	22	134
59	13	69	—	—	—	—	—	—
60	31	80	—	—	—	—	—	—
61	40	77	—	—	—	—	—	—
62	28	102	—	—	—	—	—	—
Av.	24	88	—	71	90	—	23	138

It has already been shown that sodium and potassium enter the body from the external medium. They leave the body by way of the Malpighian tubules, from which the intestinal fluid is derived. It would therefore be natural to expect that the concentration of sodium (or potassium) in the intestinal fluid would increase or decrease conformably with increase or decrease of the sodium concentration in the external medium. This is seen to be the case. If we compare the intestinal fluid of the 'NaCl larvae' with that of the 'distilled water larvae' we find that the sodium concentration is greater while the potassium concentration is the same; and if we make the comparison between the 'KCl larvae' and the 'distilled water larvae' we find conversely that the potassium concentration is greater while the sodium concentration is the same. What is unexpected is to find that in the intestinal fluid the potassium concentration is always greater than the sodium concentration, irrespective of the composition of the external medium. This is true not only of the average figures but also of the individual figures, with the single exception of serial 63, Table 3. Furthermore, in the earlier work (see Introduction), it was found for sodium that the concentration in the intestinal fluid *never* exceeded the concentration in the haemolymph; we now find for potassium that the concentration in the intestinal fluid *always* exceeds the concentration in the haemolymph. This matter receives further consideration in the Discussion.

(5) *Midgut fluid.* This is a convenient point, before discussing the composition of the midgut fluid, at which to describe certain observations which relate to the further course of the fluid which passes from the Malpighian tubules into the gut. The Malpighian tubules open into the gut in the region of the pyloric chamber, at the junction of midgut and intestine. From the pyloric chamber droplets of fluid can be seen to pass backwards, by peristalsis, down the intestine. The addition of non-diffusible dyes, such as trypan blue, to the gut contents indicates that there is a movement of fluid forwards from the pyloric chamber through the midgut to the caeca, and through the walls of the caeca to the haemolymph. So much is already known from the observations of Wigglesworth (1933*a*), confirmed by the writer (Ramsay, 1950). In the absence of any evidence to the contrary, it is natural to suppose that the fluid originating in the Malpighian tubules contributes to these two movements; but we have no idea of how the total flow is divided between the two channels and how the division is regulated.

For purposes of inserting a cannula through the anus into the intestine it is helpful to stretch the body of the larva. A chance observation suggested that the amount of intestinal fluid collected was greater if the body was kept stretched than if it was allowed to relax to a more normal length. This observation seemed worth following up; but since a stretching force applied between the respiratory siphon and the neck is not one which is normally encountered by the larva, it was decided to stretch the body by the more natural method of increasing the volume of the haemolymph.

For these experiments larvae were taken direct from the stock in tap water. The haemolymph of five larvae was collected and mixed in a cannula which was then tied into the body of a sixth larva just behind the thorax. By applying slight pressure or suction the body of the larva could be stretched or caused to collapse. The droplets passing down the intestine were counted over periods of 10 min. in the normal condition and in the stretched condition; unfortunately in the collapsed condition it was not possible to see the intestine:

	Preparation A (droplets)	Preparation B (droplets)
Stretched	10	12
Normal	0	0
Stretched	13	12

It seems that we have here a simple nervous mechanism by which the body volume is regulated. The peristaltic waves passing down the intestine are initiated and probably coordinated by the central nervous system—at least, that is a reasonable inference from the fact that no peristalsis is observed when the intestine is removed from the body and its connexions with the central nervous system are severed. Increase in the volume of the haemolymph stretches the body, and this appears to be the stimulus which initiates peristalsis; fluid passes down to the rectum and is voided through the anus. If the volume of the haemolymph is less than normal and the body wall is not under tension, peristalsis fails to occur and the fluid from the Malpighian tubules passes forwards through the midgut and is not lost to the body.

It is of some slight interest to know how far the sodium and potassium concentrations of the tubule fluid are maintained during passage through the midgut. Collections were made from the middle region of the midgut, from five larvae taken directly from the stock in tap water and placed in a medium of NaCl, 85 mM./l., KCl, 4·3 mM./l.; this fairly saline medium was used since in saline media there is greater evacuation of the solid contents of the midgut (Wigglesworth, 1933*b*). The results of the analyses are presented in Table 4, from which it can be seen that even at this short distance from the pyloric chamber there is a substantial return towards the sodium:potassium ratio characteristic of the haemolymph.

Table 4. *Composition of midgut fluid in external medium:* NaCl, 85 mM./l.; KCl, 4·3 mM./l.

(Concentrations in m.equiv./l.)

Serial	Na	K
71	171	24
72	174	44
73	230	31
74	131	18
75	117	16
Av.	165	26

(6) *Rectal fluid.* Collections of rectal fluid were made from the same groups of larvae as were used for collections of intestinal fluid. By the time the larvae were used for experiment they had remained in their various media for some 3 weeks. The analyses are presented in Table 5.

Table 5. *Effects of different external media upon the composition of the rectal fluid*

(Concentrations in m.equiv./l.)

Distilled water			NaCl, 85 mM./l.			KCl, 85 mM./l.		
Serial	Na	K	Serial	Na	K	Serial	Na	K
76	1	5	80	81	22	84	22	74
77	15	16	81	117	7	85	19	77
78	1	43	82	87	24	86	11	92
79	0	36	83	113	18	87	2·5	114
Av.	4	25	—	100	18	—	14	90

Taking first the 'NaCl larvae', and comparing them with the 'distilled water larvae', we find that the sodium concentration of the rectal fluid is greater while the potassium concentration is not significantly different. Taking the 'KCl larvae' we find, conversely, that the sodium concentration is virtually the same while the potassium concentration is greater. We can now compare the figures in Tables 3 and 5, and from them see how the composition of the intestinal fluid is altered during its passage through rectum.*

(i) In the 'distilled water larvae' both sodium and potassium are absorbed in the rectum ($P < 0{\cdot}01$).

(ii) In the 'NaCl larvae' potassium is absorbed in the rectum ($P < 0{\cdot}01$) but the concentration of sodium is not significantly changed.

(iii) In the 'KCl larvae' potassium is absorbed in the rectum ($P < 0{\cdot}01$), but only to a limited extent, while the concentration of sodium is not significantly changed.

This last conclusion is unexpected. We know from the 'distilled water larvae' that both sodium and potassium can be absorbed in the rectum, the former against the concentration gradient. In the 'NaCl larvae' it is reasonable to expect that this absorption of sodium will be held in abeyance since the element is entering the body all the time and must be got rid of. But in the 'KCl larvae' one would have expected that sodium would be absorbed and that all the potassium would be got rid of.

The difficulties of obtaining representative samples of rectal fluid have been touched upon in the Introduction; in brief, what they amount to is that under the conditions of the experiment the fluid is voided prematurely and is not kept in contact with the rectal epithelium for as long as it is normally. One cannot believe that the figures for the rectal fluid of 'distilled water larvae' are representative of the normal composition of the fluid which leaves the anus; for if the larva can live for 3 weeks in a current of distilled water—as these larvae had—and if the concentration of potassium in the fluid voided from the rectum is 25 m.equiv./l., then a simple calculation shows that all the potassium in the body would be lost if the rate of elimination was as much as 0·05 mm.^{3}/hr. The rate of collection of rectal fluid during the present experiments was undoubtedly very much greater than this, and it seems likely that the fluid did not remain in the rectum long enough for absorption to run its full course. The concentration of sodium in the intestinal fluid of the 'KCl larvae' is in any case already somewhat low, and if it has not been possible to demonstrate a further reduction of concentration in the rectum, this failure is probably attributable to the technical imperfections of the experiment. The question of the retention of potassium by the 'KCl larvae' is discussed in the next section.

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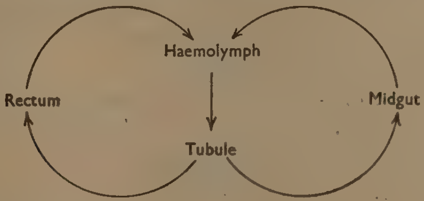
* There is no direct evidence to show whether the changes which occur in the rectum are brought about by absorption or by secretion, but there is some indirect evidence in favour of the absorption theory and this is accepted as a working hypothesis for the present.

There is no doubt that the uptake of sodium in the rectum takes place against the concentration gradient; in the case of potassium the movement is from high concentration to low concentration and there is no need to postulate any process of active transport.

DISCUSSION

The results of the experiments described in this paper have been discussed as they were presented and there is not a great deal to be added now. In outline, the sodium and potassium exchanges of the larva are straightforward. When the larva is in tap water, sodium (and potassium if it is present) is taken up by the anal gills and secreted into the haemolymph. Both sodium and potassium are present in the fluid which leaves the Malpighian tubules, and both elements are absorbed from this fluid before it leaves the rectum. When there is excess of sodium in the external medium the net inward flux is increased, the concentration of sodium in the tubule fluid rises and the absorption of sodium in the rectum is held in abeyance so that the sodium escapes from the body. The same terms can also be used to describe the movements of potassium when this element is present in excess.

But much is thereby left unsaid. It is not clear why the concentration of potassium in the tubule fluid is always greater than that of sodium even when there is no inward flux of potassium, and it is not clear why there is considerable absorption of potassium in the rectum under conditions which call for the elimination of this element. What is clear is that quite irrespective of the medium in which the larva finds itself there is a circulation of potassium within the body:



Without measurements of electrical potential to support the measurements of concentration it is not possible to establish that the movement of an ion against a concentration gradient is due to the active transport of that ion. No measurements of potential difference across the wall of the Malpighian tubule have yet been made. If, therefore, it is now suggested that the circulation of potassium is driven by some sort of 'potassium pump' operating in the Malpighian tubules, this will be understood to be no more than a tentative working hypothesis.

A similar state of affairs has been found in the Malpighian tubules of *Rhodnius* (Ramsay, 1952). In *Rhodnius*, as in *Aedes*, there is very much more sodium than potassium in the haemolymph. In the upper portion of the tubule in *Rhodnius* the fluid contains very much more potassium and less sodium than the haemolymph; in the lower portion the concentration of potassium falls and that of sodium rises so that the composition of the fluid alters towards that of the haemolymph. There is, therefore, a circulation of potassium in *Rhodnius*, comparable with that in *Aedes*.

Since the circulation of potassium in *Aedes* is not obviously adaptive in relation to ion exchanges with the external medium, and since a comparable circulation is found in the unrelated genus *Rhodnius*, one is tempted to see it as something which is fundamental to the process of urine formation in Malpighian tubules. At the moment this can be no more than speculation; but it will certainly be interesting to see how far a high concentration of potassium is demonstrable in the Malpighian tubules of other insects.

SUMMARY

1. The movements of sodium and potassium through the body and their exchanges between the body and the external medium have been studied in the larva of *Aedes aegypti* L.

2. Briefly, both elements enter the body via the anal gills, are excreted by the Malpighian tubules and can be absorbed from the tubule fluid in the rectum. When either element is present in the medium to excess its concentration in the tubule fluid is increased and the extent of its absorption in the rectum is decreased.

3. Under all conditions the concentration of potassium in the tubule fluid is greater than its concentration in the haemolymph, and the concentration of sodium is less. There is a circulation of potassium from haemolymph to tubule, to rectum or midgut and so back to haemolymph.

4. The composition of the haemolymph in respect of sodium and potassium is remarkably constant in the face of changes in the medium.

5. The volume of the larva appears to be regulated by a nervous mechanism. If the volume is increased peristaltic waves pass down the intestine more frequently and more fluid is voided through the anus.

I wish to thank Dr V. B. Wigglesworth for reading this paper in typescript and for his observations upon it.

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THE FLIGHT OF BIRDS

II. WINDING FUNCTION IN RELATION TO FLIGHT SPEED

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(With Plates 6 and 7)

In a previous paper (Brown, 1948), the movements of the wing of a pigeon in slow flight were analysed. With the help of a new photographic technique further work on the flight movements at different forward velocities has been carried out. In this paper the analysis is extended with additional information obtained from normal cine-photography. Certain improvements and simplifications in the flash cinematographic methods are also described.

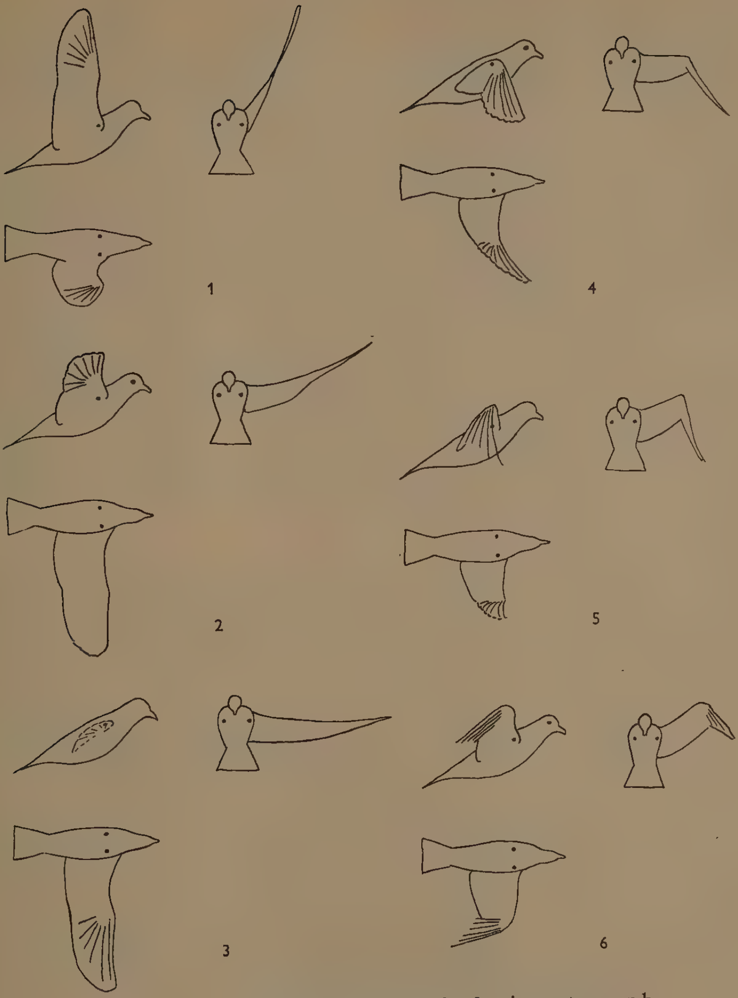
METHOD

The photographic methods are essentially similar to those previously described. The film is moved steadily past the camera lens, and the lamp or lamps are flashed at intervals such that the pictures are correctly spaced on the film. In the equipment as first used this was achieved by a complex system of rotary switches which had rather a short life. The modifications are described in the appendix.

The photographs of the fast flight were taken as the bird was flying along a 200ft. passage to its cage at one end. It was found that the pigeons could be easily trained to fly straight to the cage, and became accustomed to the lights and camera which were set up in an open doorway about 100 ft. from the beginning of the flight path.

FLIGHT ANALYSIS

The movements of the wing in slow flight have already been described in detail (Brown, 1948). It will be apparent from Text-fig. 1–6, published in the present paper, that at medium speed (20 m.p.h.) the movements of the wing are very different from those during slow flight throughout a large part of the beat cycle. The first aspect to be considered is the wing movements relative to the bird. The cycle may be taken as starting at the top of the downstroke. The wings are fully extended and are about 50° above the horizontal (Text-fig. 1). They then move downward and slightly forward through an arc of about 90° (Text-figs. 2 and 3, Text-figs. 1–6). The upstroke then starts with a slight flexure of the elbow. As the distal ends of the radius and ulna rise the wrist is flexed and the manus supinated; the primary feathers are depressed and turned backwards (Text-fig. 4). As the rise of the inner part of the wing continues it finally involves the tip feathers (Text-fig. 5), which by now are pointing backwards and are slightly spread. The rise continues until the wrist is approaching its starting point (Text-fig. 6), but before this is reached the



Text-figs. 1–6. The wing-movement cycle of a pigeon at 20 m.p.h.

Text-fig. 1. Beginning of the downstroke, wing fully extended.
Text-fig. 2. Middle of downstroke showing twisting of wing tip to give forward drive.
Text-fig. 3. Near end of downstroke, velocity is falling and angle of attack is falling.
Text-fig. 4. Beginning of upstroke. Flexure and retractions of wrist tip feathers, unstressed wrist rising.
Text-fig. 5. Middle of upstroke. Primaries are being swung violently backwards, giving a forward drive.
Text-fig. 6. End of upstroke. After the flick the primaries remain unstressed until the wing extends for the next downstroke.

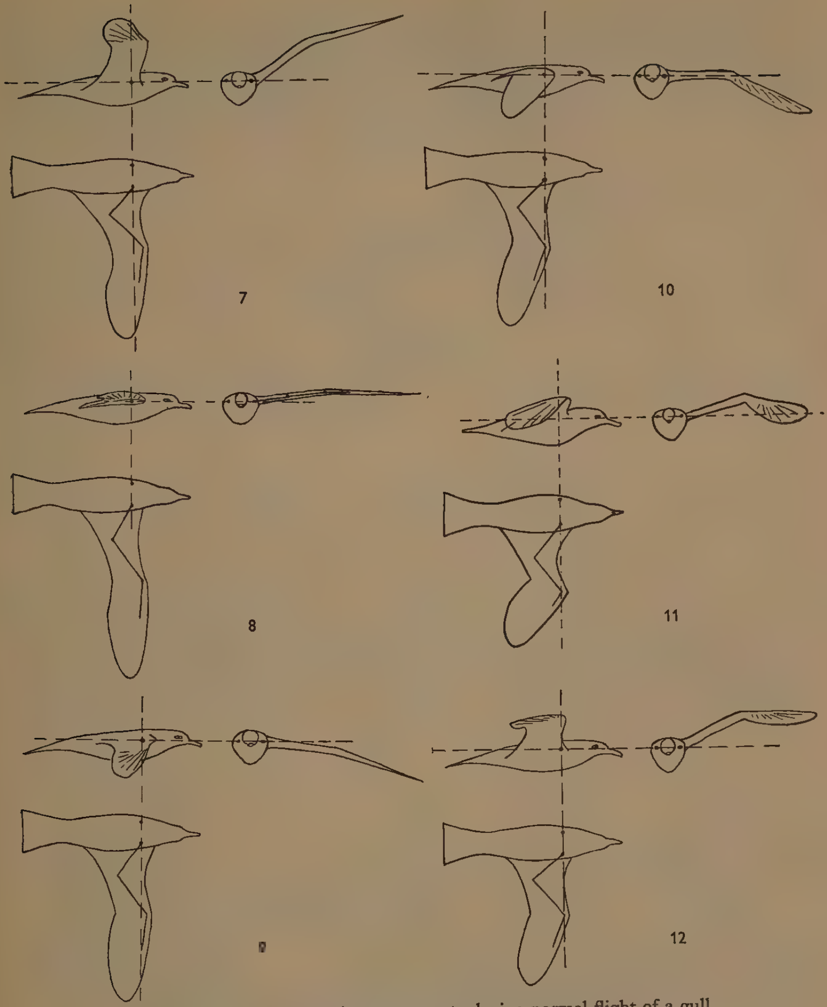
elbow and wrist are extended, the hand is pronated and the primaries spread for the next downstroke.

In this type of flight the body is nearly horizontal. The downstroke is similar to that occurring in slow flight, but the upstroke is much simpler, since there is only a slight backward flick of the primary feathers. The main muscles involved in the movements are fairly obvious. The downstroke is clearly performed by the pectoralis major. The flexure must be produced within the wing by the biceps flexure carpi ulnaris and other intrinsic flexors of the wrist and carpals. When the elbow and wrist are flexed, but not before, the pronation of the manus can take place. This movement is probably aided by air pressure on the backwardly moving tip, but is initiated by muscular action, probably by the pronator brevis.

The first effect produced when the wing moves backwards and upwards under the action of the supracoracoideus, latissimus dorsi and scapulo-humeralis; the initial stage produced is a propulsive flick of the primary feathers, while later the tip feathers are folded over each other and their angle of attack becomes zero. While the tip is undergoing this fairly complex movement the inner part of the wing is slightly flexed and rotates relatively slowly in a vertical plane round the shoulder joint. During this phase all the lift must be produced by the proximal part of the wing, which is being lifted by the air reaction; the muscles limit rather than aid this movement.

The forward movement which completes the cycle must arise from the contraction of the deltoideus, while the extension of the wing is clearly carried out by intrinsic muscles such as the triceps, patagialis group, and the extensor metacarpi radialis. At the same time this extension produces pronation in readiness for the next down-beat.

The wing movements in fast flight are very similar to those occurring at medium speed (Text-figs. 7–12 and Pl. 7) in the wing-beat cycle of a gull at high speed, which only differs from that of a pigeon in its slower rate of beat and smaller amplitude. The downstroke is simple, having only a slight forward movement (Text-figs. 7–9). The upstroke has no propulsive flick and the wing moves relatively slowly. For example, it occupies 0·5–0·6 of the cycle time as compared with about 0·2–0·3 at take-off. As the wing rises (Text-fig. 10) the inner part is only slightly flexed whilst the tip feathers are folded and retracted (there is little or no backward movement at the wrist). As this phase is completed the wing extends and the tip swings forward (Text-fig. 12). The complete cycle is therefore a simple up-and-down movement of the region of the wing lying proximally to the wrist, with a roughly circular movement of the distal region; forward and down, backward and up. The musculature involved is clear. The downstroke is produced by the contraction of the pectoralis major, and it is the only muscle involving any appreciable work. Since the air reaction is tending throughout to lift the wing, the upstroke need only be limited and controlled by muscular action, i.e. relaxation of the pectorialis major. The flexions and extensions of the wing are produced by the intrinsic muscles as described before, and since there is no propulsive flick, it requires only little muscular effort.



Text-figs. 7–12. The wing movements during normal flight of a gull

Text-fig. 7 Beginning of downstroke. The wing is fully extended and is moving with no forward component relative to the bird.

Text-fig. 8. Middle of downstroke. This shows slight twisting of the tip to give a forward force.

Text-fig. 9. End of downstroke. The wing is still fully extended and the whole is still lifting.

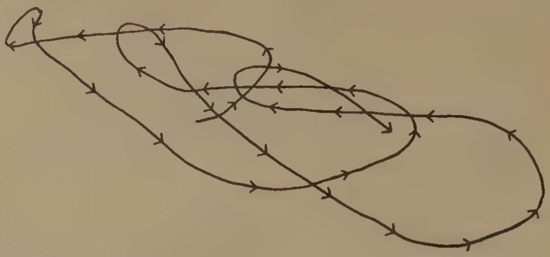
Text-fig. 10. Beginning of upstroke. Flexure at the wrist and slight retraction, increase of angle of attack of the arm to compensate for loss of tip lift.

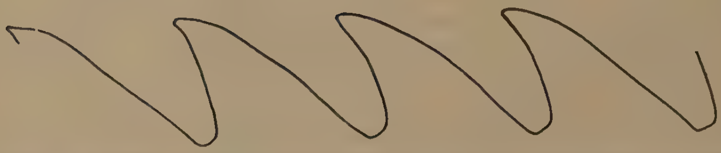
Text-fig. 11. Middle of upstroke. The primaries are folded over each other and are unstressed. Slight further retraction of wrist and increased angle of attack.

Text-fig. 12. End of upstroke. The wing is extending and the primaries are swinging forward again for the next downstroke.

Track relative to the air

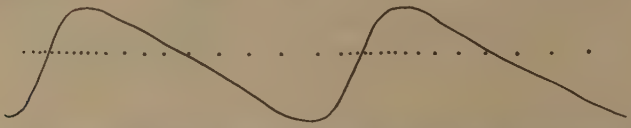
At the take-off the tip of the wing moves along a path which may be described as a series of linked figure eights (Text-fig. 13), with the forward velocity of the body remaining constant or falling as the wing moves forward and down, and then increasing as the tip travels upward and back (Brown, 1948). As the forward speed increases the backward movements decrease, and the tip track becomes opened out as shown in Text-fig. 14, and the body moves with no measurable change in velocity. In normal fast flight there is no backward movement of the tip, which traces out a simple waved track, and in birds with a slow wing beat it is possible to see a drop in body speed during the upstroke (Text-fig. 15).





Text-fig. 13. The wing-tip track of a pigeon landing. Note the very large backward and forward components of motion.

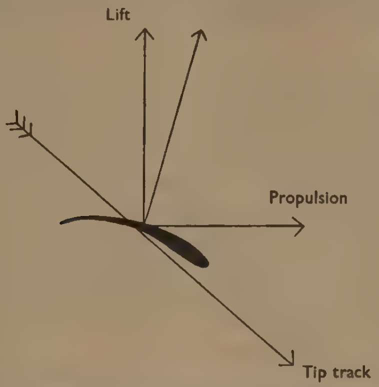
Text-fig. 14. The wing-tip track of a pigeon at 18 m.p.h. Here backward movements are greatly reduced and there is only momentary reversal of an air-stream direction on the primary feathers.



Text-fig. 15. The wing-tip track of a swan shortly after take-off. The dots indicate the position of the head in successive pictures. Note the absence of backward movement of the wing and the loss of speed, as shown by the spacing of the dots on the upstroke.

Air forces

The air reaction at take-off has been described (Brown, 1948). As the speed increases the long axis of the body tends to become horizontal and the reaction of the tip feathers during the downstroke has a forward component (Text-fig. 16). This is shown by the forward bending of the feathers in Pl. 6, fig. 6. The upstroke is propulsive during the flick, but this phase is less important than it is at take-off. Nevertheless, the tip feathers develop a propulsive component of force during parts of both the up- and downstrokes, while the inner part of the wing maintains lift throughout the flapping cycle.



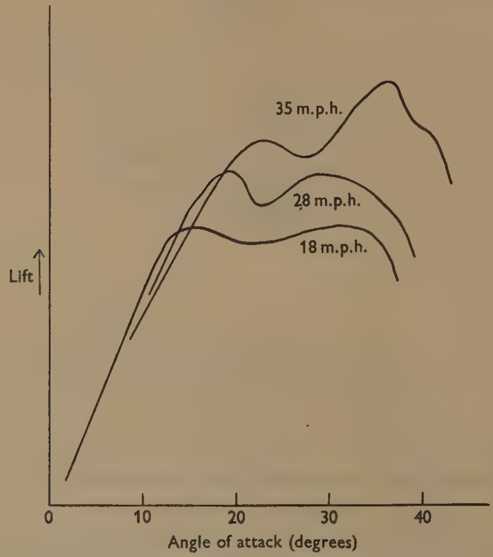
Text-fig. 16. This diagram shows how the downward- and forward-moving wing tip can give the propulsive force.

In normal fast flight it is clear from Text-figs. 7–12 and Pl. 7 that the proximal part of the wing must behave as a fixed aerofoil, while the tip provides propulsion during the downstroke only, and is passive on the upstroke.

The above account may be summarized in tabular form:

	Take-off	Medium speed	Normal high speed
Downstroke:			
Wing tip:			
Angle of attack	Positive	Positive	Positive
Angle relative to direction of flight	Positive	Negative	Negative
Function	Lift	Propulsion and lift	Propulsion and lift
Inner wing:			
Angle of attack	Positive	Positive	Positive
Angle relative to direction of flight	Positive	Positive	Positive
Function	Lift	Lift	Lift
Upstroke:			
Wing tip:			
Angle of attack	Positive (feathers inverted)	Positive (feathers inverted)	Zero or slightly positive
Angle relative to direction of flight	—	—	Positive
Function	Propulsion and lift	Propulsion and lift	Absent
Inner wing:			
Angle of attack	Positive (high)	Positive	Positive
Angle relative to direction of flight	—	Positive	Positive
Function	None	Lift	Lift

At take-off, the air speed over the inner part of the wing is very low, and the air reaction from it can contribute little or nothing to the total lift of the wing. Boel (1929) showed that this was true by cutting off the secondary feathers of a pigeon without impairing the take-off. It can be demonstrated, however, that a bird treated in this way cannot do more than fly under take-off conditions for a short time, and is apparently unable to increase its forward speed. On the other hand, trimming off part of the tip feathers prevents normal take-off; while the bird can still glide, it is unable to maintain height.



Text-fig. 17. Lift curves of the primary feather group of a pigeon at different speeds. Note the effect of the flexibility in delaying the stall at the higher wind speeds.

Wind-tunnel tests

Small birds with a rapid wing beat cannot be investigated as a whole in a wind tunnel since one cannot simulate the large velocity gradient along the wing which occurs in flight. There are, however, several features of the flight mechanism which can be studied. The experiments were designed to study the properties of the primary feather group of a pigeon. The procedure was as follows. The feathers were mounted in a wooden holder so that they occupied the positions they would have in a fully spread wing tip, and their bases were fixed into the holder by paper strips simulating the covert feathers. The complete structure was mounted in a wind tunnel and its properties measured under various conditions. Text-fig. 17 shows the lift curves of the primary feather group at different speeds in the attitude of the downstroke. It will be seen that the curve of the lift against nominal incidence varies with speed. The term nominal incidence is used because the figures cannot show the actual angles of incidence of the flexible system; the angles plotted are degrees of rotation of the wing mounting rod from the position at which the lift is zero. It is clear from the curves that the stalling is very gradual. This is not a stall as is found in rigid structure, but rather a tip incidence-limiting effect produced by the onset of stalling on the proximal part of the wing. This results in a backward movement of the centre pressure and an increased torsion, so that over the range of plotted angles above 15–20°, the actual angles of attack of the tip feathers may not increase as the wing mounting is rotated; this effect is important, in that it produces an almost unstallable wing tip.

A flexible structure such as a bird's wing can have no fixed aerodynamic properties, for these clearly change as the forces on the wing change.

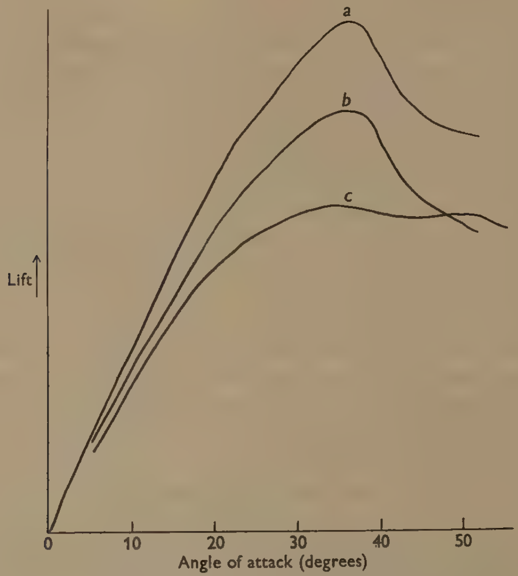
The flexible wing also compensates automatically for changes in muscular effort. If, at take-off, the wing-beat velocity is increased, the component of air speed normal to the plane of the wing is increased. In a rigid wing this would result in a greater angle of attack and might lead to stalling, but the larger forces at the higher speed twist the flexible structure and limit the angle. This is another important feature of the tip structure. In the photographs it is clear that, even though the feathers are not emarginated they separate during the downstroke when subjected to the high stresses of take-off. The properties of the wing tip in such a condition cannot be known exactly but must have the effect of preventing, or delaying, the stalling of the tip.

Measurements were made of the same group in a position simulating the backward flick of the upstroke and gave interesting results. There is no doubt that in this phase the proximal part of the wing can contribute nothing to the lift of the wing as a whole. At this stage of the flight, the tip moves backwards with the line through the feather bases, making an angle of about 90° with the air flow. The feathers separate and rotate, and each acts as a separate aerofoil. It has been suggested by Boel (1929) and others that the rotation is under muscular control. There seems no evidence that (*a*) the feather shafts can rotate and (*b*) that there is any musculature to produce such a movement; the natural elasticity of the feather can account for the observed rotation.

With the tip feathers mounted as described, they were adjusted so that the lamina of each feather rested lightly against the underside of the feather behind. The air stream was directed on to the dorsal surface, and the feathers were seen to separate as the angle of attack was increased. The curve of lift against an angle is shown in Text-fig. 18*a*.

Next, the feathers were rotated so that there was a slight gap between them when they were unstressed. This gap increased when the feathers were flexed by the air stream. The appropriate lift curve is shown in Text-fig. 18*b*; finally, they were rotated so that each lamina was pressing firmly against the next feather. The separation taking place in the air stream was small and the curve was as shown in Text-fig. 18*c*. It will be seen that the conditions of '*b*' and '*c*' both give values of lift coefficient lower than '*a*', which is as close an approximation as possible to the conditions found in the unstressed wing of the live bird.

In these experiments (carried out at an air speed of 50 ft. per sec.) neither Text-figs. 18*b* nor *c* gave lift forces sufficient to balance the weight of the bird. This fact and the observation that the curve (Text-fig. 18*a*) gave the highest L/D ratio, together suggest that the feathers separate by virtue of their own elasticity and not by muscular activity. The low L/D ratio (reaching a maximum in these tests of about 4) may be of little importance, since the wing tip is moving through the air in a direction opposite to the direction of flight, and its drag is therefore in fact a propulsive force.



Text-fig. 18. Lift curves of the primary feathers of the pigeon with the wind directed on to the dorsal surface. (For explanation see text).

DISCUSSION

In a previous paper (Brown, 1948) it was pointed out that the take-off flight therein described was not to be considered as normal flight since it was not impaired by the removal of the secondary feathers (Boel, 1929).

It was suggested that the differences to be expected between slow and fast flight were: (*a*) disappearance of the backward flick, and (*b*) transfer of the propulsive function to the downstroke. This has now been confirmed and evidence presented which shows that as the speed increases so the flick of the tip feathers gradually decreases and the body tends to approach in a horizontal position. The downstroke becomes nearly vertical and of smaller amplitude and develops a greater forward component of reaction from the tip feathers.

There is general agreement on the function of the wing on the downstroke in fast flight; the inner part gives lift while the tip provides propulsion. Concerning the wing function at take-off there is some divergence of view. Storer (1948) describes what he calls the 'helicopter' and 'variable propeller', used in take-off, and 'reversed propeller' in landing. The author states that the wing-tip feathers can be rotated so as to provide either a backward or forward force, or, in other words, that they can have two stable positions in relation to the air-stream direction. I cannot accept this statement. I am convinced that the feather cannot receive an air stream at an angle of greater than about 15° without becoming unstable. If a single feather is examined in a wind tunnel it will be found that above the angle of maximum lift the feather becomes unstable and flutters violently. At angles below the maximum it has a configuration similar to that illustrated by Storer for the normal state, but the configuration labelled as in reverse flight cannot be seen. I believe, therefore, that under all conditions and speeds of flight, the downstroke is carried out with the tip feathers at a fairly small angle of attack.

During take-off and landing the tip moves along a path whose general direction is against the direction of flight, the reaction from the tip being upward with probably slight forward inclination at the take-off, and a backward one during landing.

The upstroke is much more complex, and the disagreements in the literature are numerous; in part due to the fact that there are a number of different types of wing movements which can be carried out by the same bird, and also that not all birds move their wings in the same way in similar situations.

Several authors (Headley, 1912; Marey, 1890; Horton Smith, 1938; Guidi, 1938) all believe that the primary feathers separate on the upstroke to reduce resistance.

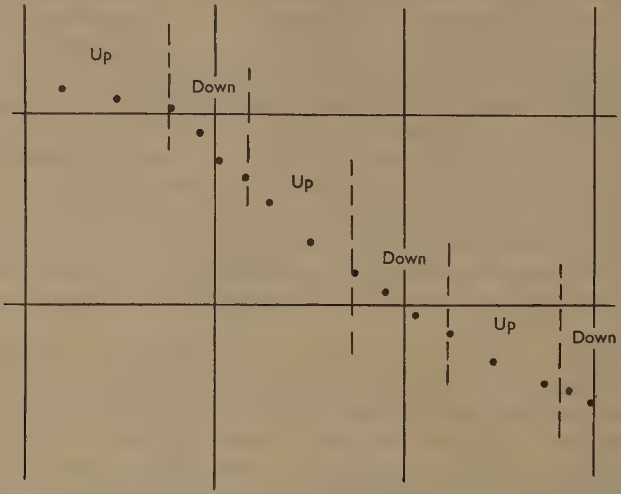
Aymar (1936) suggests that a backward push may be produced, and shows photographs in which the primary feathers are bent forward. Horton Smith (1938) has stated that there is a downward and forward force. This is also the opinion of Storer (1948). Lorenz (1933) on the other hand, believes that the rotation of the feathers produces a series of overlapping aerofoils whose combined reaction is forward and upward.

This is also suggested by the work of Marey (1890) who showed that in the flight of a duck there were two periods of vertical acceleration in the beat cycle.

From inspection and analysis of photographs I am convinced that the view of Lorenz (1933) is correct. During slow flight the upstroke clearly provides propulsion, without loss of height; in fact there is a gain in vertical velocity during climbing flight (Text-fig. 19).

When the flight speed rises after take-off and the body angle approaches the horizontal, the tip flick disappears. At the same time the speed of movement of the wing falls and the tip is carried up passively. There is no evidence in my photographs that the reaction of the air stream on the tip feathers ever has a downward component, as suggested by Horton Smith (1938) and others, and I find it difficult to believe that so inefficient a condition with an upward force on the secondaries and a downward force on the primaries would ever occur except as a momentary transitional phase. It is true that, as Guidi (1938) states, the feathers separate, but I suggest that this action increases lift at take-off rather than reduces air resistance.

Under all conditions where a propulsive upstroke is not used, the tip feathers are folded over each other, rather than separated when the wing is raised; the air stream is never directed against their dorsal surface.



Text-fig. 19. Plot of the movement of a pigeon in climbing flight. The dots represent the position of the shoulder in successive pictures; note the increase in speed during the upstroke.

APPENDIX

Photographic apparatus

In the course of this work on bird flight it was necessary to develop a flash cinematographic technique which has been of more general use.

An early form of this equipment has been described (Brown, 1945), and the following is an account of a greatly improved version. The basic principle is simple. The object to be photographed is illuminated by a series of flashes of light emitted from special lamps and the reflected light is focused on to a film moving at such a rate that the pictures are correctly spaced along it. The duration of a flash is so short that no appreciable movement of the film takes place within it.

The various units are as follows: camera, drive and flash control switching, low-power pulse generator, power unit and main pulse generator, lamp circuits, monitor and test circuits.

Camera

The camera is a modified hand-cranked 35 mm. cine-camera. The modifications are:

(*a*) Removal of the shutter and intermittent motion.

(*b*) Fitting an additional sprocket to pull the film through the gate.

(*c*) Replacement of the hand-crank by a coupling to engage with the drive unit.

The absence of shutter is not essential, but if present is only of use in limiting the effect of extraneous light at low camera speeds. In the particular camera its inertia would limit the starting acceleration so that the full speed of 90 pictures per second could not be reached in under half a second. Without the shutter the full speed is reached in about $\frac{1}{10}$ sec.

Mounting and drive

The camera is clamped to a board on which is mounted the drive unit. The unit comprises a $\frac{1}{4}$ h.p. electric motor which drives a free pulley on a shaft. This pulley can be locked to the shaft by a clutch. This clutch is operated by a solenoid which at the same time releases a spring-loaded brake. Also on the shaft are: (1) a commutator with two opposite contact bars which perform the flash control switching, and (2) a coupling to engage with the camera shaft.

The drive unit is controlled by a double-circuit push button which is connected by a flex lead to the board. When the button is pressed down against a light spring pressure it makes the motor circuit and the motor starts. Further movement against a heavier pressure completes the solenoid circuit, releases the brake engaging the clutch and starts the camera. The clutch and brake system enables the camera to be run up to full speed in about $\frac{1}{10}$ sec., and stopped in rather less time. This rapid operation is of great value, since it is possible to take shots lasting only $\frac{3}{4}$ sec. or less, with about 65 pictures containing only about 9 pictures at the beginning and 6 at the end, where the time intervals are not uniform.

Low-power pulse generator

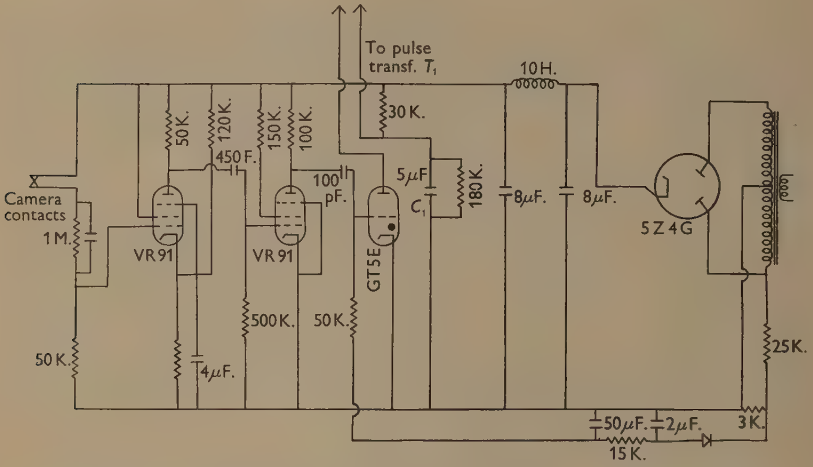
The circuit is shown in Text-fig. 20, and consists of a pulse generating circuit operated by the commutator on the camera drive. The gas-filled relay, GT 5 E, feeds a pulse from C into the pulse transformer T_1, which is a car ignition coil with a new secondary winding. The output is a 15 kV. pulse which operates the lamp circuits.

Power unit and main pulse generator

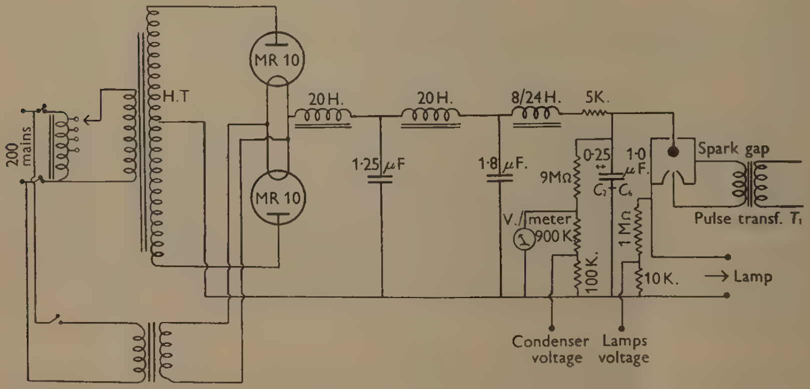
The power has a simple biphase rectification system with choke input smoothing, and can deliver 10,000 V. d.c. at 0·5 A. (Text-fig. 21). This feeds the flash condensers C_2–C_6 through a choke and resistance to limit the charge rate. These condensers and the triggered spark gap form the high-power pulse generator. When the spark gap (Wilkinson, 1946) receives a pulse from the ignition coil it breaks down and feeds the stored energy of 12–50 joules to the lamp or lamps. The flash is varied by using one or more of the condensers C_2–C_6 in parallel.

Lamp circuits

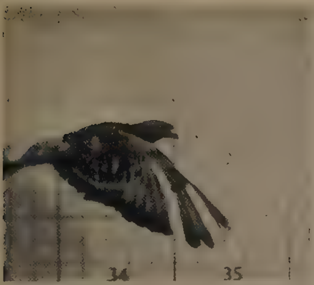
The lamps used are B.T.H. type F.A.I. which were intended to be used at 2000 V. and flashed by a triggering pulse applied to a wire on the outside of the envelope. When fed with a 10 kV. pulse they flash without being triggered. The advantage of using 10 kV. is that for the same power much smaller condensers, which can be charged quickly between flashes, can be used. When using a single lamp it is simply put in series with the spark gap, with a small condenser in parallel with it. Two lamps are normally used, connected in series. The condensers allow the spark gap to maintain ionization by charging the condenser while the voltage builds up on the lamp to the flash level.



Text-fig. 20. The circuit of the low-power pulse generator. For description see text.



Text-fig. 21. Diagram of the main power unit and flashing circuit. The points labelled condenser voltage and lamp voltage are used with an oscilloscope for checking the function of the equipment.



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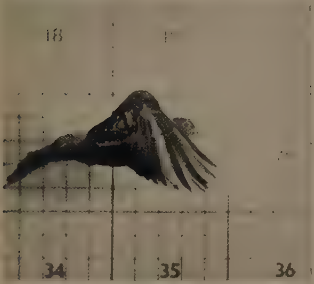

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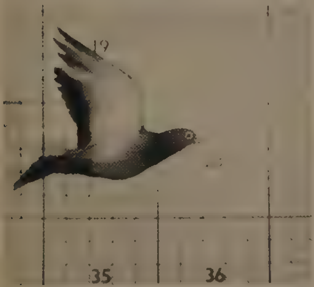

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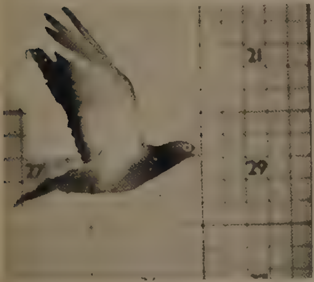

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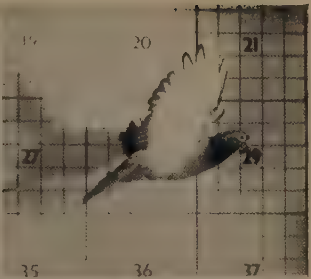

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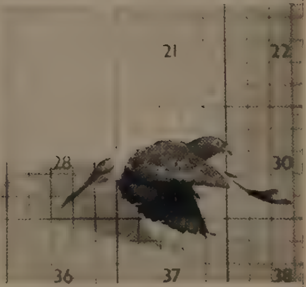

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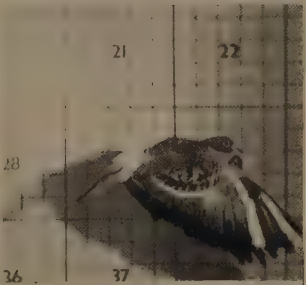

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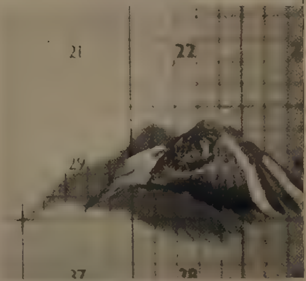

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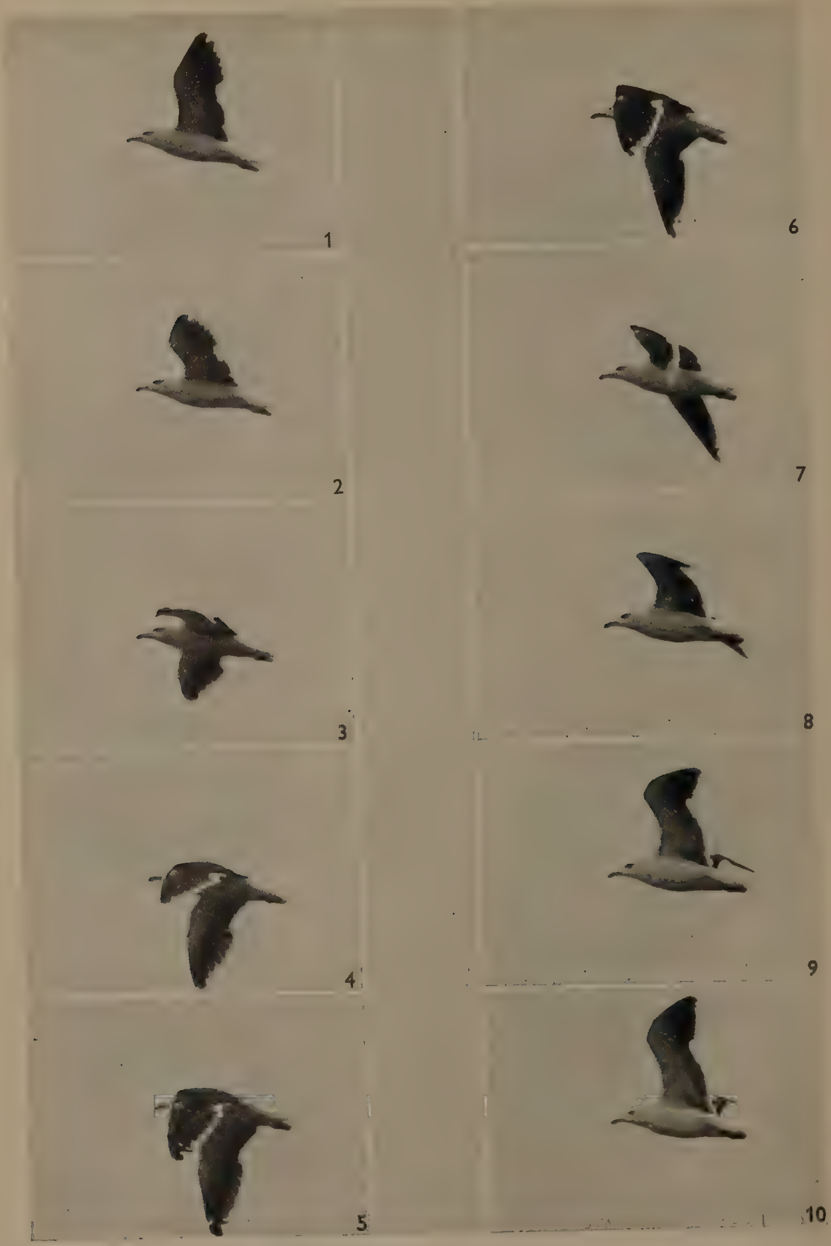
BROWN—THE FLIGHT OF BIRDS



BROWN—THE FLIGHT OF BIRDS

SUMMARY

1. The changes in the form of the wing movement of different birds with change of air speed are described.

2. The aerodynamic properties of various configurations of the wing are discussed.

3. Reconciliation of various contradictory accounts in the literature is attempted.

4. Improvements in photographic methods are described.

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EXPLANATION OF PLATES

PLATE 6

Pigeon in flight at medium speed, showing slight forward bending of the feathers during the upstroke (fig. 2) and again in the downstroke (fig. 6).

PLATE 7

Gull in normal fast flight. No forward bending of the tip feathers in the upstroke (figs. 6–8), and little reduction of area of the inner part of the wing.

THE INFLUENCE OF TEMPERATURE AND MOISTURE ON THE UPTAKE AND LOSS OF WATER IN THE EGGS OF *GRYLLULUS COMMODUS* WALKER (ORTHOPTERA—GRYLLIDAE)

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I. INTRODUCTION

In almost all studies on the gain and loss of water by the eggs of insects, changes in the wet weight of the whole egg have been used as a measure of changes in water content. This procedure is admissible in the case of water uptake because the error introduced by the weight of the dry matter used in oxidative processes during development is always small compared with the changes in water content that occur. Furthermore, most insect eggs, being cleidoic, require no substances other than water from the environment, so that no error is introduced in this way (Needham, 1942). In the case of desiccation, the error introduced by the oxidation of dry matter is positive but may be neglected provided the changes in weight are fairly large in relation to the original weight, or if the period during which such changes occur is short.

Most authors, when studying changes in the water content of eggs, have estimated the average changes in the water content by weighing a number of eggs together. The method is convenient because a fairly large weight can be obtained and changes in weight are more easily measured. It suffers from the great drawback, however, that the variability in the behaviour of individual eggs during changes in water content cannot be measured. Since such individual variations are often important, it is preferable to weigh individual eggs, as has been done by Salt (1949 *b*).

II. MATERIALS AND METHODS

The eggs used in this work were laid by some hundreds of crickets kept together in a large cage. The eggs were laid in trays of moist sand which were removed daily and new ones replaced. On removal of the trays the sand was sieved under water and the eggs washed, sorted and either used immediately or stored at 13° C. on wet blotting paper in an airtight jar.

During the experiments the eggs were kept in small numbered 'pockets' made in plaster disks which were either saturated with water when the experiment required a saturated atmosphere for the eggs, or were in equilibrium with the air in the desiccators when desiccating conditions were used. Thirty eggs were placed on one disk which was kept in an airtight jar in which the humidity was controlled with distilled water or sulphuric acid solutions. Temperatures were maintained constant within ±0·1° C.

Individual eggs were weighed at daily or more frequent intervals on a torsion balance of 0–50 mg. capacity, calibrated in units of 0·02 mg. Care was taken during weighing to exert as little pressure on the eggs as possible, particularly when water was being lost and the eggs had become flaccid.

III. RESULTS

(*a*) *Uptake of water*

When the eggs of *Gryllulus commodus* are incubated soon after laying some develop and hatch promptly but others enter a diapause stage. Diapause is most rapidly and efficiently completed during an exposure to low temperature (Browning, 1952*a*). The proportion of eggs which manifest diapause and the intensity of the diapause is influenced by the environment of the eggs. For example, when eggs are incubated at a high temperature (about 30° C.) few enter diapause; at lower temperatures, (about 25° C.) most eggs enter diapause; whilst when temperature is quite low (about 20° C.), diapause occurs in virtually all the eggs. In all cases, however, an adequate exposure to an appropriate low temperature (13° C.) soon after laying results in the eggs becoming competent to complete their development and hatch promptly when incubated at higher temperatures. Nevertheless, an egg that is to be incubated at a low temperature (say 20° C.) requires to be exposed to 13° C. for a longer period than one that is to be incubated at a higher temperature (say 30° C.) if it is to have an equal chance of completing its development without diapause (Browning, 1952*b*).

(i) *Eggs incubated within 24 hr. of the time of laying*

In Fig. 1 is shown the course of increase in mean weight in eggs incubated on moist plaster at each of three constant temperatures, using thirty eggs at each temperature. Sigmoid curves of weight increase similar to these have been found for a number of insects (Banks, 1950; Johnson, 1937; Roonwal, 1936; Salt, 1949*b*).

The rate of water uptake, as measured by the time required by the eggs to reach the point where their weight ceased to increase significantly, was more rapid at higher temperatures than at lower. A similar situation was shown by Banks (1950) in the eggs of *Corixa*, and by Salt (1952) in the eggs of *Melanoplus*.

At 29·9° C. development was erratic. Out of a total of thirty eggs, thirteen hatched, whilst four entered diapause and eight did not increase in weight after the initial period of slow increase in weight. The remaining five eggs were injured during handling and died after having taken up their water. These eggs are very easily damaged at the time when water uptake is nearing completion, frequently bursting whilst being dried prior to weighing. This was not observed except at this temperature. At 26·5° C. only two eggs did not increase in weight beyond the initial stage, and one of these was injured on the second day. Only two eggs in this group hatched, the remainder entered diapause. At 20·9° C. six eggs did not increase in weight and all the remainder entered diapause.

(ii) *Eggs in which diapause development had been completed*

Eggs which had been stored at 13° C. for a period after laying were placed on moist plaster at each of the three constant temperatures, 29·9, 26·5 and 20·9° C., thirty eggs being used at each temperature.

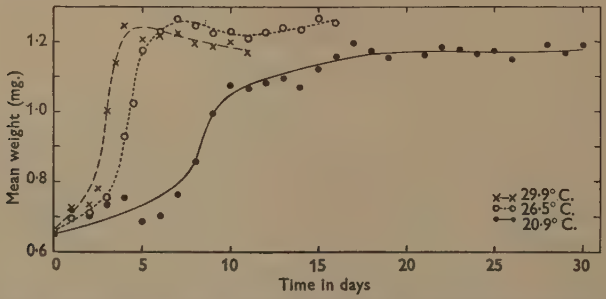

Fig. 1. Showing the changes in the mean weight of eggs during incubation at three different constant temperatures and incubated without preliminary exposure to low temperature.

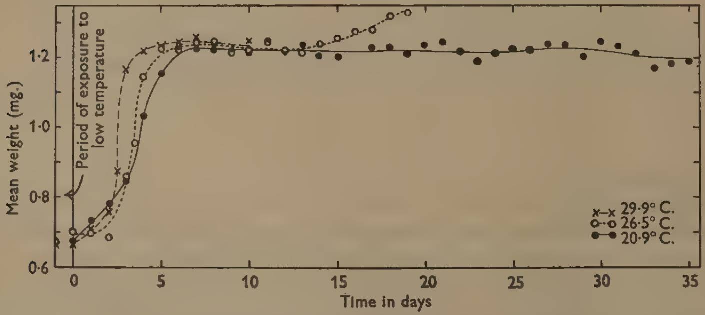

Fig. 2. Showing the changes in the mean weight of eggs during incubation at three different constant temperatures following a period of exposure to low temperature.

Those eggs which were to be incubated at 29·9° C. were held at 13° C. for 15 days, the corresponding period for eggs to be incubated at 26·5 and 20·9° C. being 17 and 39 days respectively. This was necessary to ensure that all eggs were comparable with respect to diapause at the beginning of the observations of water absorption (Browning, 1952*b*). Since over 90 % of the eggs hatched and there were no significant differences in the numbers emerging at the different temperatures, it was considered that this object had been achieved. The eggs increased in weight slowly whilst remaining at 13° C., so that those that were to be incubated at 20·9° C. were significantly heavier at the beginning of the incubation period than those in the other two treatments.

Fig. 2 shows the course of water uptake at the three temperatures which are very similar to those shown in Fig. 1 for newly laid eggs. The fact that the curve for eggs at 20·9° C. is not more markedly different from the other two may be explained by the fact that almost the whole of the initial period of slow water uptake had been completed by these eggs at 13° C., whilst this was not so for the other two groups, which had spent a shorter time at low temperature.

The chief difference between the eggs that had been exposed to low temperature before the incubation and those that had not was found in their variability. The former were much less variable than the latter, both with respect to the initial period characterized by a slow increase in water content and with respect to the rate at which the water content increased during the period of rapid increase. This was particularly true of the eggs incubated at 20·9° C. The decreased variability in eggs which had been exposed to low temperature before they were incubated is illustrated by the smoothness of the curves in Fig. 2 compared with those of Fig. 1.

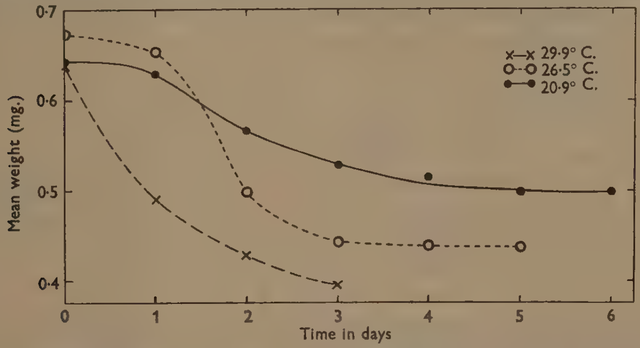

Fig. 3. Showing the trend in the rate of loss of water from newly laid eggs under similar conditions of atmospheric saturation deficiency at three different constant temperatures.

Observations made both on living eggs and on eggs which had been fixed and stained failed to reveal any special area which might control the uptake of water, such as is found in grasshopper eggs (Slifer, 1938; Steele, 1941).

(*b*) *Loss of water*

(i) *Newly laid eggs*

Three groups of twenty eggs each were placed on plaster disks in airtight jars containing sulphuric acid solutions whose concentrations were adjusted so that the saturation deficiency of the air in the jars would be 3·226 mm. of mercury at each of the three temperatures, 20·9, 26·5 and 29·9° C. The eggs were weighed each day, and the curves of Fig. 3 show the mean weights of each group during the period of observation. The variation in weight between individual eggs at any temperature at any one time was small, and so this method of presentation of the data has been used here (see § III (*b*) (ii) below).

During weighings it was noticeable that after the eggs had reached their lowest mean weight they began to increase in weight significantly during the following few days. This was attributed to the growth of fungi within the eggs rather than to an uptake of water by the eggs themselves.* Only the descending parts of the curves are shown in the figure.

The eggs lost weight most rapidly at the highest temperature, although the saturation deficiency of the air was similar at all temperatures. This contrasts with the view put forward by Mellanby (1935). The differences seem too great to be accounted for by the increased rate of diffusion of water vapour at the higher temperatures.

The eggs in these experiments were exposed to relatively moist air (between 82·5 and 90 % relative humidity, depending upon the temperature). In view of the rate at which the newly laid eggs lost water (Fig. 3) they must be considered extremely susceptible to desiccation. In nature the eggs are laid in heavy black clay soil which usually remains sufficiently moist throughout the winter and spring to enable many eggs to survive.

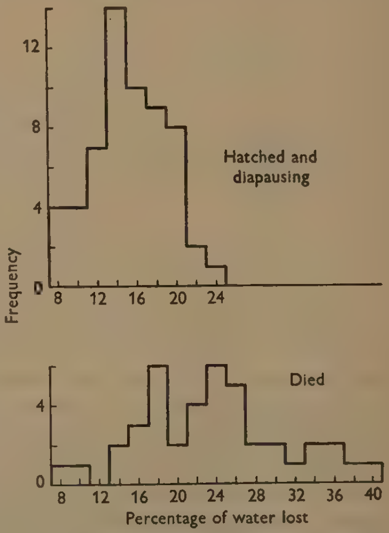

Fig. 4. Histograms showing the percentage of weight lost during a period of desiccation by fifty-nine eggs that survived the treatment and forty-one eggs that subsequently died.

The eggs in these experiments, when replaced on moist plaster after 5 or 6 days, showed no sign of absorbing water and were evidently dead. An experiment was then done to determine how much water the eggs could lose without dying.

One hundred newly laid eggs were weighed and placed in an atmosphere of 90 % relative humidity at 29·9° C.; they were removed after 18–26 hr., weighed and placed on moist plaster at 29·9° C. Observations were made of the eggs that subsequently hatched or entered diapause and of those that died.

In Fig. 4 are shown frequency histograms representing the percentage of the original weight lost during desiccation, of the eggs that either hatched or entered diapause and of those that died. It can be seen that the loss of more than about 20 % of the original weight was likely to result in an egg's death, whilst most eggs were able to withstand a loss of up to about 20 % of their original weight with no apparent harmful consequences.

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* Fungi do not seem to influence directly the viability of eggs. High percentages of eggs were observed to hatch even when they were completely covered with fungal hyphae after long periods of storage at low temperatures. Dead eggs, however, seem to be readily invaded by hyphae.

(ii) *Eggs which had completed water uptake*

Three groups of thirty eggs which had been stored for 70 days at 13° C. and had reached a weight greater than 1·0 mg. were placed at 90 % relative humidity at 29·9, 26·5 and 20·9° C., and each egg was weighed daily until its weight fell below 0·6 mg. At this point they were considered to be dead and were discarded. This limit was chosen because, in a large series of similar experiments, no egg was found to recover after its weight had fallen to 0·6 mg. When the records of weight loss were examined it was found that at each temperature, individual eggs had lost weight at such different rates that the mean daily weight of the group was of little use in describing the trend of weight loss. This became emphasized after the first 3 days when many eggs had been discarded, leaving only a few quite heavy eggs to contribute to the means.

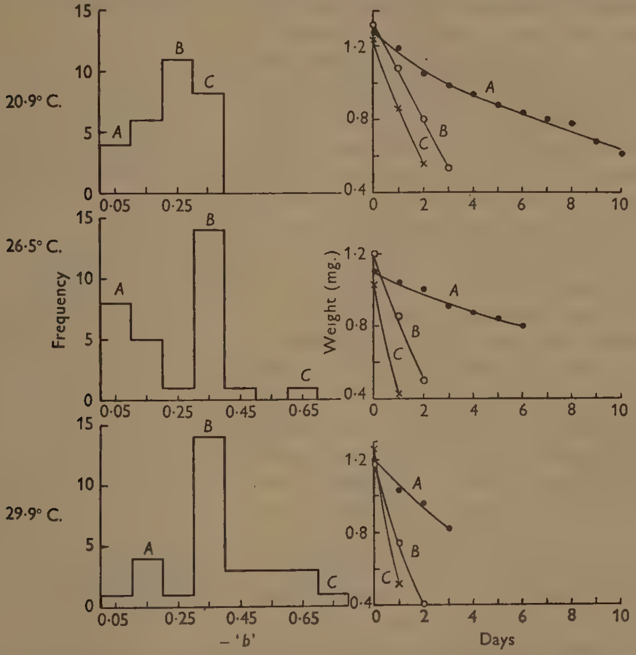

Fig. 5. Histograms showing the distribution of rates of loss of water, b, within samples of thirty eggs at each three constant temperatures and curves showing the actual course of water loss from representative individuals from groups labelled similarly in the corresponding histograms.

For this reason the regression of weight lost against time was calculated for each egg. Fig. 5 shows the distribution of b, the linear regression coefficient. Although the weight of an egg is not a linear function of the duration of exposure, yet it was considered that the linear regression, by accounting for a substantial part of the total variance, would provide an adequate statistic to enable comparisons to be made between individual eggs at the same temperature and between groups of eggs at different temperatures.

In Fig. 5 the right-hand set of diagrams shows the course of weight loss of representative individuals from groups labelled similarly in the corresponding histograms. The curves were drawn free-hand through the observed points.

At each temperature two distinct kinds of eggs may be distinguished, one which lost water very slowly and one which lost water quite rapidly. These do not seem to be merely the extremes of a very variable population, but rather to be representative of distinct populations which differ in their resistance to desiccation. When comparing eggs at one temperature with those at another, some of the differences—the loss of distinction between the two kinds of eggs at 20·9° C. for example—may have been due to the increased power of desiccation of the air at higher temperatures when the relative humidity was maintained constant.

In order to estimate the stage during desiccation at which eggs lost the power to reabsorb water and continue development to the point of hatching, 100 weighed eggs, which had completed water uptake and diapause development during storage for 70 days at 13° C. were desiccated at 90 % relative humidity and 29·9° C. for periods varying from 24 to 48 hr. At the end of this time the eggs were again weighed and transferred to moist blotting-paper at the same temperature. At the end of 12 days most of the eggs had either hatched or died, but a few remained turgid and appeared healthy but showed no visible signs of development. These eggs appeared to be in a state resembling diapause, even though they had received an exposure to low temperature estimated to be sufficient to enable them to complete diapause development.

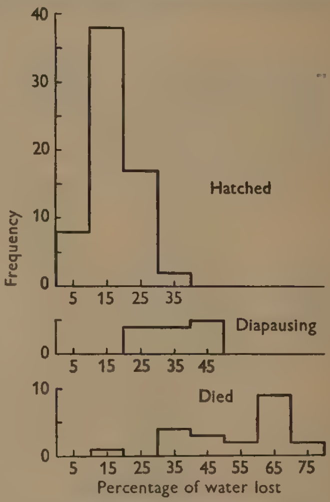

Fig. 6. Histograms showing the percentage of weight lost during a period of desiccation at 29·9° C. by eggs which had completed water uptake at 12·8° C. Sixty-five eggs survived the treatment, twenty-one died and thirteen entered a state resembling diapause.

Fig. 6 shows the frequency distribution of the percentage of weight lost by eggs that either hatched, entered diapause or died.

In order to study the unusual diapause which had occurred in these eggs the following experiment was done. Some hundreds of eggs, which had completed water uptake during an exposure of 4 months to 13° C., were desiccated at 90 % relative humidity at 29·9 C. for 56 hr. They were then returned to moist plaster and left until most of the hatchings were complete. 100 eggs were then carefully selected which were fairly turgid and which appeared, under a binocular microscope, to be in diapause. Twenty-five of these were placed at 29·9° C. as controls, and the remainder, in three lots of twenty-five eggs each, were placed at 13° C. At the end of 15, 25 and 35 days, twenty-five eggs were removed from the cold and placed at 29·9° C., and observations of hatchings were made each day.

This experiment gave no significant results in so far as the numbers of nymphs hatching in the four treatments were concerned. However, although about the same numbers of nymphs hatched in each treatment (between one and three) the numbers of eggs which developed to the point of having clearly visible eye-spots and cerci were considerably greater in the group of eggs stored at low temperature for 35 days compared with the controls. A number of embryos, particularly in the cold-treated groups, were found to have developed to the point where revolution was about to occur but to have been unable to undergo the movement round the posterior pole of the egg. In these cases morphogenesis had continued so that 'monsters' were produced, in which the development of the external form of the embryo was complete, or almost so, but the embryo was facing the posterior instead of the anterior pole of the egg. Similar monsters occurred in which the embryos underwent about half their revolution, and fully developed embryos bent backwards on themselves were found. In still other cases development continued to the point where hatching should normally occur, the embryo became darkened and hardened, but was unable to escape from the egg-shell. These cases are similar to those described by Andrewartha (1943) in eggs of *Austroicetes* which had been exposed to low temperatures for inadequately long periods to permit the completion of diapause development. It can be concluded then that these eggs of *Gryllulus* had been induced to enter a state closely resembling diapause. But it would seem best to regard this as a pathological condition because diapause is not naturally induced in *Gryllulus* in this way and because the treatment necessary to promote the further development of these eggs was not the same as that necessary to promote the completion of the normal diapause development which occurs in *Gryllulus* eggs.

(iii) *Eggs at different stages of development*

A large number of eggs which had been kept for 3 months at 13° C. and had completed water uptake during this period were placed at 29·9° C. on moist plaster. After the eggs had been at 29·9° C. for 0, 1, 3, 5, 7 and 9 days, samples of thirty eggs were taken and placed at 90 % relative humidity and 29·9° C. and each egg was weighed daily. The results are set out in Fig. 7, which was prepared in the same way as the histograms of Fig. 5.

The eggs, which initially lost weight rapidly, became more resistant to desiccation up to about the fifth day and then became less resistant. Similar changes have been shown for the eggs of *Austroicetes* by Birch & Andrewartha (1942) and for eggs of *Smynthurus* by Davidson (1932).

No eggs hatched among the thirty that were desiccated immediately after transfer to high temperature, nor in those that were incubated for 1 day. Two eggs hatched of those that had 3 days' incubation, ten of those that had 5 days, nine of those that had 7 days' incubation and none in the sample that was incubated for 9 days prior to desiccation. All the eggs that hatched lost water slowly. However, a number of eggs in the 0- and 1-day samples lost water quite as slowly as those that hatched in later samples, yet they had so much development to undergo that they died before completing it. The only cases in this experiment in which eggs hatched whose rates of water loss were greater than 0·099 mg. per day (those that did not fall within the first group interval of the histograms of Fig. 7) occurred among the sample that was incubated for 7 days initially and thus had only about 3 days development to complete. Even in these cases, however, the greatest rate of water loss among the eggs that hatched was 0·147 mg. per day, and most rates were much less than this. In all cases hatching occurred at least 1 day later than would be expected for eggs developing at the same temperature (cf. Birch, 1944).

A striking difference can be seen between the histograms for 0 day initial incubation in Fig. 7 and that for the loss of water from eggs at 29·9° C. which had completed water absorption at 13° C., shown in Fig. 5. The only difference in the treatment of these two groups of eggs was in the time spent at 13° C. The eggs in Fig. 7 were in a slightly more advanced stage of development than those of Fig. 5, having spent some 20 days longer at 13° C., and it can be seen that their modal rate of water loss was less rapid. Morphogenesis proceeds very slowly at 13° C. (eggs kept for a year at this temperature did not complete more than one-third of their morphological development), and this rather rapid development of the capacity to resist water loss in eggs exposed to this low temperature suggests that the two processes (morphogenesis and the capacity to resist water loss) may respond in quite different ways to changes in temperature.

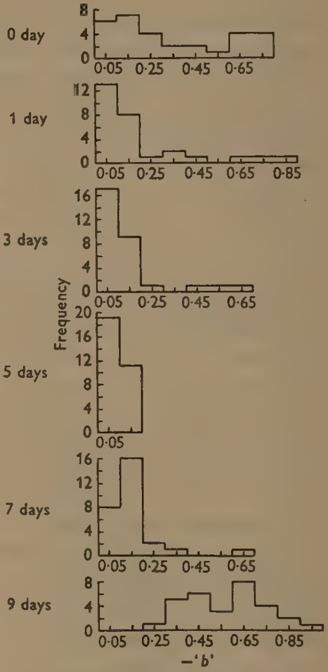

Fig. 7. Showing the distribution of the rates of loss of water, *b*, in samples of thirty eggs which had completed water uptake during storage at 12·8° C., and which were desiccated at intervals after removal to 29·9° C. for incubation.

IV. DISCUSSION

In the development of eggs of *Gryllulus commodus* there was an initial period during which the weight of the eggs increased slowly, and this was followed by a period when the water content of the eggs increased rapidly. Similar changes have been observed for other species whose eggs absorb moisture during development. The changes in water content must be an expression of physiological changes taking place in the eggs during development. The nature of these changes is not understood, but it is known that the stage of development is important, since in *Gryllulus*, *Corixa* (Banks, 1950) and *Melanoplus* (Salt, 1952) the time after the egg is laid at which the change from slow to rapid increase in water content occurs is dependent upon the incubation temperature, being shorter at higher temperatures. There is evidence also that in *Locustana* water uptake occurs only when the egg has access to oxygen (Matthée, 1951). The osmotic pressure of the egg contents may also be important.

The course of increase in water content was similar, irrespective of whether the eggs entered diapause or developed without interruption. It would seem that the eggs either took up their water before entering diapause or water uptake continued during diapause. In most eggs that have been studied the period of rapid water uptake occurs either before the eggs enter diapause (Salt, 1949*a*) or is delayed until after diapause is complete (Birch & Andrewartha, 1942; Slifer, 1946). It would be unusual for most of the water uptake to occur during diapause, and this suggests that in *Gryllulus commodus* diapause supervenes after water uptake is complete.

In a number of species the resistance to water loss from the egg is associated with the presence of one or more layers of lipoid material in the egg shell (Beament, 1946, 1951; Matthée, 1951; Salt, 1952; Slifer, 1948). Salt (1952), however, has shown in *Melanoplus* that, after this mechanical waterproofing has been completed, the egg, as it develops, continues to change with respect to the rate at which water passes into it and out of it. Similarly, in *Gryllulus* the way in which the eggs become first increasingly and then decreasingly resistant to the loss of water when exposed in dry air suggests some mechanism, at present unknown, other than simple mechanical waterproofing.

The great variability in the resistance to water loss found within samples of eggs which had been treated similarly was comparable with the situation found by Salt (1952) in *Melanoplus* eggs. More must be known about the processes governing water exchange in insect eggs before an explanation of this situation will be possible.

The inception of a state resembling diapause, following the loss of a considerable proportion of their water, by eggs in which diapause development had been completed at low temperature was similar to that described by Salt (1947) for the larvae of *Cephus cinctus* that were exposed to high temperature. Such a phenomenon implies that the physiological state which is brought about gradually at low temperature and renders the organism competent to continue its morphological development, can be destroyed by unfavourable environmental conditions.

SUMMARY

1. Eggs placed on moist plaster took up water slowly at first and then more rapidly until they had reached their maximum water content. The duration of the initial period of slow water uptake was dependent upon temperature, becoming shorter as the temperature was raised. Similarly the duration of the period of rapid water uptake decreased with temperature.

2. No special water-absorbing structure was identified in the eggs of *Gryllulus*.

3. The rate of water loss from newly laid eggs was more rapid at higher than at lower temperatures under conditions of constant atmospheric saturation deficiency.

4. Newly laid eggs died if they lost more than about 20 % of their original weight.

5. At 90 % relative humidity, eggs which had completed water uptake lost water at a higher rate at high than at lower temperatures. The rate of water loss was also dependent on the stage of development of the embryo.

6. Eggs that had completed diapause development at 13° C. and had completed water uptake were able to lose about 30 % of their weight through desiccation without being killed, but many of those that lost more than about 20 % of their weight failed to develop when replaced on moist plaster; it seemed as if they had entered a state resembling diapause.

The author wishes to thank Dr H. G. Andrewartha for much helpful discussion and interest during the course of this work. Miss Margaret Morphett prepared the figures, and she and Miss Colleen Hill assisted greatly in the routine work and the calculations.

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SOME ASPECTS OF THE MECHANICS OF MAMMALIAN LOCOMOTION

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Gray (1944) analysed the pattern of muscular activity in mammals, but there is practically no published experimental data which could be used to check this analysis. Elftman (1938, 1939*a*, *b*, *c*) working with man, and Manter (1938), working with the cat, developed various types of apparatus for recording the forces exerted on the ground, but Manter's published results give adequate data for the calculation of patterns of muscular activity for a single stride only. Following similar work on the Amphibia (Barclay, 1946), an attempt was therefore made to record the forces exerted on the ground by various mammals and to obtain synchronous photographs of the limb positions with a view to calculating the activity in the main groups of limb muscles.

The apparatus used was a development of that employed with Amphibia (Barclay, 1946) and is shown in Fig. 1. Its main advantage over previous types of apparatus is that it practically eliminates rotation and tilt, and there is therefore no need to ensure that the feet are placed centrally on the platform.

The experiments were carried out on three dogs of different types, two goats and two sheep. All the records conformed generally to the same type, and although some variation was found in detail in individual strides, the general pattern of forces at the feet was constant. In a normal stride only one limb at a time was actually on the platform. An example is given in Fig. 2, where the axis of the limb was taken as the line drawn between the centre of the part of the foot applied to the ground and the centre of articulation of the limb with the body. In the hindlimb the centre of articulation is the centre of the acetabulum, and its position was easily fixed in photographs in relation to bony prominences on the body. In the forelimb the glenoid cavity moves considerably because the scapula moves against the body; here the centre of articulation was taken to be the centre of rotation of the scapula, and this was harder to fix exactly in the photographs.

The photographic records showed the position of the limbs and the forces exerted on the ground at the same moment in three axes at right angles. From the angle of inclination of the limb and the vertical force (i.e. proportion of the weight) exerted on the ground at any stage it is possible to calculate the longitudinal forces which would be exerted on the ground if the limb were a rigid strut and the extrinsic limb muscles were not active. This horizontal force may be called the horizontal strut effect or horizontal effect of the weight. In this series of experiments the photographs did not show the angle of inclination of the limbs laterally but only longitudinally. Calculations could therefore only be made on the horizontal forces in the longitudinal axis, and the horizontal forces referred to in the discussion are all longitudinal horizontal forces.

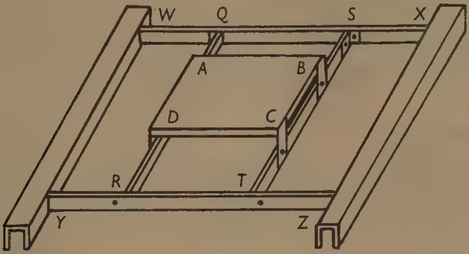

Fig. 1. The wooden platform *ABCD* is let into a larger platform (not shown), over which the animal walks. *ABCD* is fixed rigidly to a pair of steel bars *QR* and *ST*, ¼ in. × 1½ in. × 3 ft. These in turn are bolted to a pair of similar steel bars *WX* and *YZ*. These are welded to a metal base which is screwed to the floor. The movements of *ABCD* in three axes at right angles are recorded, by means of three straw levers, on a smoke drum. By means of a mirror this is placed in the same photographic plane as the animal so that photographs of the animal taken from the side include a record of the forces at the same moment.

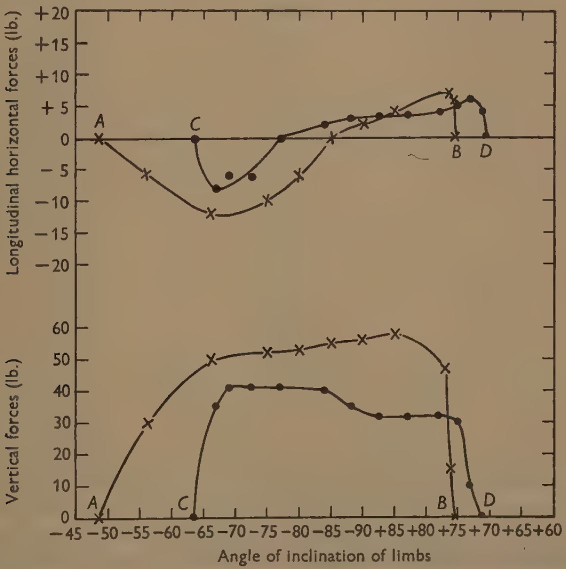

Fig. 2. Force records for a normal stride in a dog weighing 92 lb. The line *AB* represents the forces exerted on the ground by the left forelimb and the line *CD* those exerted by the left hindlimb. The angle of inclination is the angle made by the limb as a whole with the ground, negative when the foot is in front and positive when it is behind the centre of articulation of the limb. Negative longitudinal forces are forces contrary to the direction of movement.

The difference between the calculated horizontal effect of the weight (H_W) and the horizontal force actually recorded at the foot (H_R) is the horizontal effect of the activity of the extrinsic limb muscles (H_M) or, as it has been called, the horizontal lever effect. Clearly $H_R = H_W + H_M$. The force records when co-ordinated with the photographs of the limb positions therefore give a measure of the activity in the extrinsic limb muscles. Since the height above the ground of the centres of articulation of the limbs with the body did not vary greatly, H_M is a fairly accurate measure of the total couple exerted on the limb by its extrinsic muscles.

Table 1. *Forelimb*

Angle made by limb with the ground $=\alpha$ $-$protraction $+$retraction	Weight resting on limb $= W$ in lb.	Horizontal component of weight $H_W = W \times \cot\alpha$	Recorded horizontal force $= H_R$	Horizontal force due to extrinsic muscles $H_M = H_R - H_W$: Postive $=$ retractor couple	Horizontal force due to extrinsic muscles $H_M = H_R - H_W$: Negative $=$ protractor couple
		Dog			
−56	30	−20·0	− 6	+14·0	
−66	50	−22·3	−12	+10·3	
−75	52	−14·0	−10	+ 4·0	
−80	53	− 9·3	− 6	+ 3·3	
−85	55	− 4·8	0	+ 4·8	
90	56	0	+ 2	+ 2·0	
+85	58	+ 5·1	+ 4		−1·1
+77	47	+11·0	+ 7		−4·0
+76	15	+ 4·0	+ 6	+ 2·0	
		Goat			
−82	70	− 9·8	− 6	+ 3·8	
−87	72	− 3·8	0	+ 3·8	
+89	72	+ 1·3	+ 4	+ 2·7	
+83½	70	+ 8·0	+ 8	0	
+78½	68	+14·0	+12		−2·0
+75	63	+17·0	+15		−2·0
+70½	40	+14·2	+12		−2·2
+70	10	+ 3·6	+ 2		−1·6
		Sheep			
−74	40	−11·4	− 4	+ 7·4	
−77½	70	−15·2	−16		−0·8
−82	100	−14·0	−16		−2·0
−86½	100	− 6·1	− 4	+ 2·1	
+88	105	+ 3·7	+ 4	+ 0·3	
+85	98	+ 8·6	+ 8		−0·6
+80	92	+16·2	+14		−2·2
+74	82	+23·5	+20		−3·5
+69	45	+17·2	+10		−7·2

A series of typical calculations of this type on the fore and hind limbs of a dog, sheep and goat are shown in the Tables below. In both the fore and the hind limbs a constant pattern of muscular activity was observed, except that the exact point at which the change took place from a retractor to a protractor couple varied within narrow limits, probably in accordance with the degree of acceleration or deceleration of the animal; it was, however, always shortly after the foot had moved to be behind the centre of articulation of the limb. The records are for normal walking.

A few records were also obtained with goats standing with one foot on the platform. Normally the goat always stands with its two fore feet in front of the scapula and the two hind feet behind the acetabulum. In this position the horizontal forces at the feet were smaller than the horizontal component of the weight (H_W) and demonstrated a small retractor couple on the forelimbs and a small protractor couple on the hindlimbs. This is the same pattern of muscular activity as that shown in walking.

Table 2. *Hindlimb*

Angle made by limb with the ground $= \alpha$ − protraction + retraction	Weight resting on limb $= W$ in lb.	Horizontal component of weight $H_W = W \times \text{cotan}\,\alpha$	Recorded horizontal force $= H_R$	Horizontal force due to extrinsic muscles $H_M = H_R H_W$ Positive = retractor couple	Horizontal force due to extrinsic muscles $H_M = H_R H_W$ Negative = protractor couple
		Dog			
−67	35	−14·8	− 9	+ 5·8	
−69	41	−15·7	− 6	+ 9·7	
−72½	41	−13·0	− 6	+ 7·0	
−77	41	− 9·5	0	+ 9·5	
−84	40	− 4·2	+ 2	+ 6·2	
−88	35	− 1·2	+ 3	+ 4·2	
+87½	32	+ 1·4	+ 3	+ 1·6	
+83	32	+ 3·9	+ 3		−0·9
+78	32	+ 6·8	+ 4		−2·8
+75	30	+ 8·0	+ 5		−3·0
+73	10	+ 3·1	+ 6	+ 2·9	
+71	0	0	+ 4	+ 4·0	
		Goat			
−72	15	− 4·8	− 2	+ 2·8	
−73	25	− 7·7	− 4	+ 3·7	
−77	45	−10·4	− 6	+ 4·4	
−79	55	−10·7	− 6	+ 4·7	
−82	55	− 7·7	− 6	+ 1·7	
−84	50	− 5·2	0	+ 5·2	
−86	50	− 3·5	+ 2	+ 5·5	
+88½	50	+ 1·3	+ 4	+ 2·7	
+84	50	+ 5·2	+ 6	+ 0·8	
+81	50	+ 7·9	+ 8	+ 0·1	
+78½	50	+10·2	+10		−0·2
+74	50	+14·6	+12		−2·6
+72	50	+16·2	+14		−2·2
+69	47	+18·0	+14		−4·0
+67	30	+12·8	+ 8		−4·8
+65	15	+ 7·0	+ 2		−5·0
		Sheep			
−73	27	− 8·3	− 4	+ 4·3	
−77	42	− 9·7	− 8	+ 1·7	
−79	50	− 9·7	− 8	+ 1·7	
−82	55	− 7·7	− 8		−0·3
−86½	65	− 4·0	− 4	0	
−89	72	− 1·3	+ 2	+ 3·3	
+87	70	+ 3·7	+ 6	+ 2·3	
+83	60	+ 7·4	+ 9	+ 1·6	
+78½	62	+12·6	+12		−0·6
+74½	62	+17·2	+12		−5·2
+71	30	+10·3	+ 5		−5·3

The lateral forces were generally less than the longitudinal forces, but since there was no record of the lateral inclination of the limbs, no calculations could be made of the pattern of adductor or abductor muscle activity.

These results could be summarized by saying that a group of extrinsic muscles is active (i.e. exerts a couple greater than its antagonists) when the foot is on the opposite side of the proximal limb joint to the origin of the muscles concerned. The exception to this is that for a short time after the feet are behind the joint the retractors are still active in both fore- and hindlimb. This, however, only lasts while the feet are so nearly vertical that the actual horizontal forces remain low. The pattern of muscular activity is therefore always such as to maintain the horizontal forces at the feet at a very low level. Usually it reduces them to a figure very considerably below the horizontal component of the weight or the horizontal force which would be developed by any other pattern of muscular activity. These generalizations are not affected by certain erratic readings such as those shown in the tables for the dog and sheep.

Calculation on results given by Manter (1938) for the cat, and Elftman (1940) for man, show the same pattern of extrinsic muscular activity. It is also the same as that shown to be present in the toad and newt (Barclay, 1946) and demonstrated by Gray (1944) and Barclay (1946) to be in almost every respect the most efficient possible mechanically.

SUMMARY

1. An apparatus is described for measuring the forces exerted on the ground by mammals in three axes at right angles.

2. Analysis of these force records and synchronous photographs of the limb positions shows a constant pattern of activity in the protractor and retractor muscles of both limbs.

3. This pattern of activity is basically the same as that previously demonstrated in the toad and newt and shown to be mechanically in most respects the most efficient possible.

I wish to thank Prof. J. Gray for constant advice and help and Mr K. Williamson for technical assistance. The work was done while holding a Coutts-Trotter Research Studentship at Trinity College, Cambridge.

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POTASSIUM IONS AND INSECT NERVE MUSCLE

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INTRODUCTION

A number of analyses of the haemolymph of insects have been made by different authors using direct chemical and colorimetric techniques or flame-photometry. These analyses show that the haemolymph from different types of insects varies enormously in ionic composition. Furthermore, the values obtained diverge considerably from the relatively constant compositions of the blood of vertebrate animals, and even from the figures commonly obtained from a variety of other invertebrates. The ratios of the principal ions present in both vertebrates and most invertebrates are similar in general proportions to those in sea water of the present day, as was first pointed out by Macallum (1910). For the purposes of the present paper interest is confined to the ions of sodium and potassium. These are present in sea water in the ratio Na:K, 50:1. In frogs the blood ratio is 40:1 and in man it is about 28:1. In most invertebrate animals the ratio falls between 25:1 and 50:1 (data from Prosser, 1950). In the insects, however, the ratio determined has often been much lower than these values and may be less than unity. Some extreme values are collected together in Table 1; further examples may be obtained from the sources referred to therein. Very low Na:K ratios are not found in all insects, and in some the ratio is nearer to that found in vertebrates. Two such examples are included in the table; the values for frog blood are given for comparison.

In an extensive series of analyses Boné (1944), demonstrated that the low Na:K ratio in insects is associated with the herbivorous habit. The normal ratios are found only in carnivorous insects, and intermediate values are found in insects with an omnivorous diet. Attempts have been made to depress the ratio in omnivorous forms by feeding them on exclusively vegetarian diets containing a high concentration of potassium. Tobias (1948*a*) was able to increase the serum potassium of *Periplaneta americana* by 56 % after changing from an omnivorous diet to lettuce, which contained less sodium and more dry-weight potassium. He was not, however, able to depress the ratio below unity by this procedure. Tobias adds in a footnote a personal communication from Boné. The latter found no effect of diet on haemolymph Na:K ratio in *Tenebrio molitor*. He apparently concluded 'that the ionic composition is a species characteristic, maintained by a mechanism of mineral regulation'. Successful depression of the ratio to a level just below unity was nevertheless achieved by Ramsay (1952), in the blood-feeding bug *Rhodnius prolixus* by adding potassium chloride to the blood diet. These data are interesting in themselves and raise many problems concerning mineral regulation and diet, but they present a much more serious problem to the nerve physiologist. The knowledge that vertebrates need to maintain a constant blood composition by active regulation has been known for a century and led to the famous Claude Bernard dictum 'La fixité du milieu intérieure est la condition de la vie libre'. The insects referred to are capable of surviving considerable changes in blood composition. In Ramsay's experiments normal responses were obtained from a bug whose haemolymph potassium concentration was five times the concentration which is lethal to vertebrates. Mellanby (1939) has regarded insect blood as a reservoir of water, quoting evidence that the blood may become reduced in volume by as much as 70 % by water loss. He makes no mention of the physiological consequences which one would expect to find in many animals following such a change.

Table 1

Animal	State	Diet	Na (as mM./l)	K (as mM./l)	Author
(D) *Pegomya* sp.	Larva	Leaves	26	58	Boné (1944)
(L) *Ephestia kuhniella*	Larva	Meal	17	60	Boné (1944)
(O) *Stenobothrus stigmata*	Adult	Leaves	61	62	Boné (1944)
(L) *Sphynx pinastri*	Pupa	—	Trace	35	Brecher (1929)
(L) *Pieris brassica*	Pupa	—	Trace	37	Brecher (1929)
(L) *Cecropia* sp.	Larva	Leaves	4	49	Drilhon (1934)
(L) *Sphynx ligustri*	Larva	Leaves	3	47	Drilhon (1934)
(L) *Prodenia eridanea*	Larva	Leages	13	39	Babers (1938)
(L) *Bombyx mori*	Larva	Leaves	14	40	Babers (1938)
(C) *Cicindela maritima*	Adult	Insects	162	9	Boné (1944)
(O) *Gryllotalpa vulgaris*	Adult	Insects	174	11	Boné (1944)
Man			143	5	From Prosser (1950)
Frog			104	2·5	From Prosser (1950)

(D) Diptera. (O) Orthoptera.
(L) Lepidoptera. (C) Coleoptera.

The most pressing problem, however, concerns the irritable tissues, to which attention was drawn by Katz (1949). It is now well established that resting nerve and muscle membranes are in a state of polarization which disappears and partially reverses when the membrane is excited. The propagation of a wave of depolarization constitutes the nerve impulse, and local or propagated disturbances of similar type precede muscle contractions. The resting potential difference across the membranes of both nerve and muscle is maintained by active processes involving ion transfer (see review by Hodgkin, 1951, and Hodgkin and Huxley, 1952). A net result of this transfer is the maintenance of a difference of potassium-ion concentration between the inside and outside of the nerve and muscle membranes. The concentration is much greater on the inside of the membranes than in the surrounding body fluid of all cases studied. The resting potential is clearly associated with the difference in potassium-ion concentration, for an increase in the external potassium-ion concentration causes a progressive decrease in this potential. The decline is slight over a small range of potassium concentration starting at zero, but soon becomes logarithmic. The potential is zero at a concentration of about

130 mM. K/l. The curves giving the relationship between the resting potential and external potassium concentration are remarkably similar in squid giant axon, frog sartorius muscle and frog myelinated nerve (Hodgkin, 1951). Some of the recorded potassium concentrations in the haemolymph of herbivorous insects are so large (Table 1) that one would expect these nerve and muscle tissues to be almost completely depolarized in such a medium. Under these circumstances the nerve would not propagate an action potential and the muscle contractile mechanism could not be excited. A further and related issue concerns the sodium concentration. The maintenance of the action potential also demands the presence of a certain minimal quantity of sodium ions on the outside of the membrane (Hodgkin, 1951). In the haemolymph of the larvae of *Sphynx pinastri* and *Pieris brassica*, Brecher found only traces of sodium. Tobias found very little sodium in *Bombyx mori* larvae at pupation.

The potassium problem has been given two possible explanations. Heilbrunn (1943) proposed that the insect potassium is largely bound to organic molecules and is not, therefore, free to depolarize the membranes. Hodgkin (1951) suggested that insects may have evolved some other basic mechanism for producing the resting and action potentials than the classical one of the crab, squid and frog. The present paper describes investigations on the effect of potassium ions on nerves and muscles of *Locusta migratoria migratorioides* R. & F., with a view to establishing the extent to which the nerve and muscle membranes of a typical insect diverge from the classical examples in their relation to these ions. These insects are normally herbivorous and were reared and fed in the laboratory on common British grasses. They will, however, eat other foods when hungry, and become cannibals under conditions of extreme starvation.

NERVE PREPARATIONS

In the earliest experiments ventral nerve cords were dissected out and mounted on silver electrodes. They were stimulated maximally at one end and the action potential was recorded near the other. These cords did not live long in any of the saline media used, and the action potential diminished quite rapidly when the potassium concentration in the bathing medium was raised to 70 mM./l. They survived much longer when simply exposed *in situ*, and it was early observed that survival time increased with decreasing extent of dissection. This was probably related to the necessity of maintaining the tracheal system intact, as was stressed by Pumphrey & Rawdon-Smith (1937), in regard to their cockroach preparations, although it is notable that Adrian (1931) kept *Dytiscus* isolated nerve cords alive for 2 days, and Roeder (1948) has more recently obtained continued spontaneous activity in isolated cockroach nerve cords over periods up to 60 hr. The changes in the multiple spike potential of the cord with changing potassium were not easy to interpret. It was clearly desirable to obtain a single nerve-fibre preparation *in situ* and one which could be obtained with very little damaging dissection. This proved quite easy to obtain, and depends on the observations that the muscles of arthropods are innervated by only a few fibres (Pringle, 1939; Wiersma, 1941). The fine branches of the motor nerves, usually carrying only two fibres, pass down the centre of each of the muscle units into which the muscles are divided and branch frequently to supply individual muscle fibres at several places. (Details of histology and nerve supply will be published later.) Thus, if finely tapering electrodes, insulated down to the tips (about 10 μ diameter), are inserted into a muscle unit, and finely graded square-wave stimuli are applied, a single motor fibre can usually be excited and fired antidromically. The action potential can be picked up from the whole nerve trunk some distance away. In the experiments described below, a unit of the flexor tibialis muscle was used to provide the appropriate nerve site.

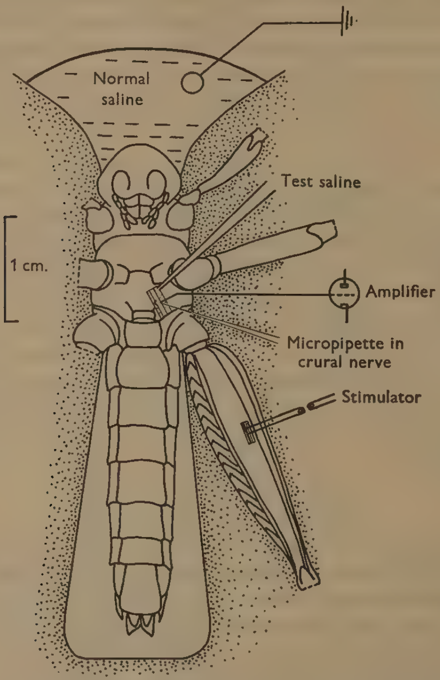

Fig. 1. Sketch of preparation showing positions of electrodes and pipettes. The stippled area shows the extent of the plasticine couch.

Animals were decerebrated through a hole cut in the top of the head and laid, ventral surface uppermost, in a couch of plasticine which held them in position without the use of pins. The hole also served to make contact with a pool of saline which was earthed and used as a reference point. The abdomens were left completely free so that tracheal pumping was not interfered with (Fig. 1). A small piece of cuticle was removed from between the inner ventral ridges of the femur, exposing part of a unit of the flexor tibialis muscle. The stimulating electrodes were then placed in position. A piece of the ventral tergite of the metathorax was removed, together with the underlying epithelium, exposing the edges of two large air-sacs. These were separated to reveal the crural nerve which supplies the motor fibres of the flexor tibialis muscle below, and an insulated silver-hook electrode was placed under this. A pipette was inserted through the gap by the electrode to direct test salines for bathing the whole nerve trunk. Many preparations were ruined through autotomy, but an equal number survived. Stimuli were derived from a square-wave generator and were passed through an isolating transformer to reduce stimulus artefact. The action potentials were recorded with respect to earth via a direct-coupled amplifier. The potentials were recorded after first sucking away fluid surrounding the nerve with a pipette. They were triphasic, with the first phase positive as the impulses emerged from the leg which was at earth potential. The spike then swung negative as the impulse passed the recording electrode and then gave a final small positive phase. The negative phase was about 100 μV. and conduction velocity 2·2–2·7 m./sec. at 20 °C.

Various salines were tried in early experiments following the compositions of Chauvin (1941), Bělăr (1929), and Pringle (1938), both with and without added glucose. The ultimate procedure was to prepare two mixtures, each containing the same calcium, magnesium, phosphate, bicarbonate and chloride, but one with 140 mM. K/l. and 10 mM. Na/l., the other with 150 mM. Na/l. and no potassium. No glucose was used in the mixtures. These were mixed together in various proportions from stock so that a variety of potassium concentrations from 0 to 140 mM./l. could be obtained quickly. Each mixture had the same osmotic strength and none had less than 10 mM. Na ions per litre. Mixtures with specific Na/K ratios were kept in a thermostatic bath and fed to the pipette in the preparation. A basic mixture was used for the saline pool making contact with the body via the hole in the head. This mixture had the following composition per litre of water.

K	10 mM.,	H_2PO_4	6 mM.,
Na	140 mM.,	HCO_3	4 mM.,
Ca	2 mM.,	Cl	150 mM.
Mg	2 mM.,		

The experimental test salines had the same calcium, magnesium, phosphate, bicarbonate and chloride as this, but the Na/K ratio was varied as described above.

Nerve preparation results

The stimulus strength was increased until a single spike just appeared, and the trunk was then bathed for some time in the normal saline. This was then replaced by the test saline and spike height plotted against time. The test saline was circulated gently round the nerve trunk. Some typical results are shown in graphical form in Fig. 2. Each curve was obtained from a single locust. With a potassium concentration of 70 mM./l. or less there is little change in the potential over 6 hr. With higher concentrations the spike height gradually diminishes, usually reaching a fairly steady level a few hours after the start of the experiment. The steady level is reached more slowly, and has a lower final value, as the potassium concentration is increased. Potassium ions do, therefore, reduce the nerve potential, at least in high concentrations. Alternatively, as Pringle suggested (personal communication), the nerve sheath, which has not been adequately described in insects, may simply be acting as a very efficient barrier to the passage of ions. To test this possibility, a micro-syringe device was prepared so that salines could be injected through the sheath. The device consisted of narrow plastic tubing filled with oil and connected to a hypodermic syringe at one end and to narrow glass tubing at the other. Glass pipettes with 3–5 μ tips, ready filled with test saline, could be quickly attached by shellac to the glass tubing and mounted on a micro-manipulator. A small hole was first bored through the sheath just above the recording electrode with a fine glass needle. The tip of the pipette was then inserted and saline injected. 70 mM. K saline injected in this way reduced the negative phase of the spike to zero in a few seconds. 50 mM. K regularly produced an equal block and even 40 mM. K did so on one occasion. Saline with only 5 mM. K had no effect on the action potential. When the blocked region was subsequently bathed in low-potassium external saline the second phase of the action potential reappeared slowly. With very large in-

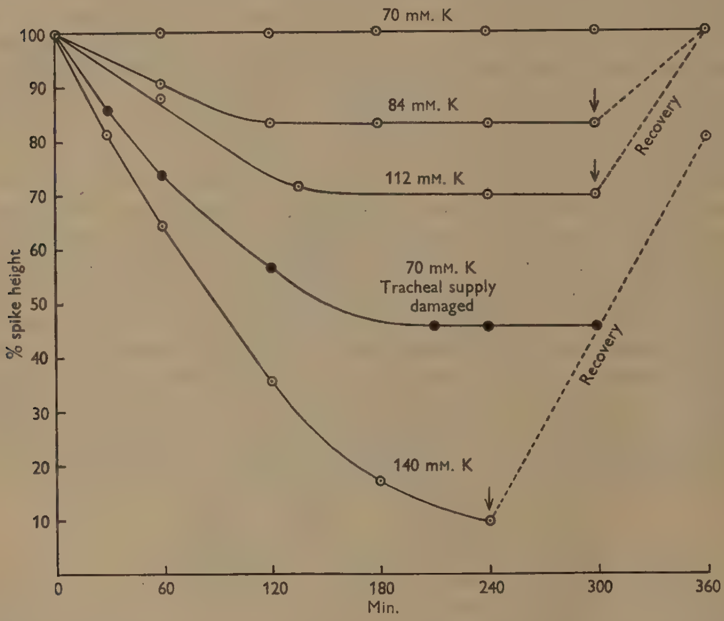

Fig. 2. The effect of high potassium concentrations in the bathing fluid of the crural nerve on the spike height of a single motor axon. The dotted lines indicate recovery following bathing in 10 mM. K starting at the arrows. Five individual locusts giving typical results.

jections it was possible to reduce the size of the positive phase of the spike in addition, even to obliterate it, and recovery was then very slow and sometimes incomplete.

The block developed more slowly when only small volumes of 70 mM. K were injected, and it was thus possible to follow stages in the building up of the block. After a few seconds, as potassium ions diffuse across to the active fibre, the local potassium concentration increases and partially depolarizes the nerve. This process produces a characteristic delay in the negative spike (see Bullock & Turner, 1950), which also appears reduced in height. The delay increases progressively until the blocking concentration is reached over a sufficient length of nerve, when the negative spike completely disappears. Later, as the potassium ions further diffuse down the nerve trunk and mix with the existing interaxonic fluid, the local concentration decreases again and the reverse process occurs (Hoyle, 1952). The delayed, small, negative spike potential suddenly reappears and increases gradually with reducing delay, until the action potential is normal again about 2 min. after the injection. It is evident, therefore, that the axons are depolarized by similar concentrations of potassium ions to those which depolarize crustacean (Cowan, 1934), squid (Curtis & Cole, 1942) and frog myelinated nerve (Huxley & Stämpfli, 1951), and that, as in these preparations, depolarization is very rapid when the saline is brought into direct contact with the axons. The sheath surrounding the locust crural nerve axons must act as a highly efficient diffusion barrier to potassium ions. This has been further confirmed by dissecting away small patches of the sheath (complete removal proved too difficult), in the vicinity of the recording electrode, and adding a local drop of high potassium saline. Blocking occurs almost immediately, and conduction is equally rapidly restored by washing the treated region in potassium-free saline (Fig. 3).

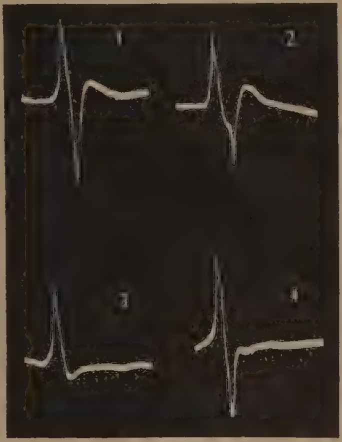

Fig. 3. The single fibre action potential, (1) and the effect of applying a drop of 70 mM. K saline to a point near the electrode following local removal of the sheath, (2) after 10 sec. showing partial depolarization, (3) after 60 sec. showing complete block in conduction, (4) after washing in 5 mM. K saline.

The question arises as to whether the sheath is an active or a passive barrier or both. It was noticed that in some preparations 70 mM. K saline applied to the whole nerve trunk did, in fact, reduce the nerve potential. This phenomenon appeared to be correlated with damage to the tracheal system, so the latter was deliberately damaged by cutting the tracheal trunks supplying the crural nerve, and the time course of reduction of the potential was measured. The potential now declined relatively rapidly in 70 mM. K saline (Fig. 2). This could be due to an effect on the axons themselves, owing to the depletion of their air supply, but this should also have affected their excitability, and this was not appreciably changed. It is also possible that the damage reduced the efficiency of the sheath. The latter covers the whole nervous system including the brain, the other ganglia and the peripheral nerves right down to the muscle insertions. It consists of three layers: an outer very thin tracheolated membrane, a thick structural non-cellular neural lamella which is laminated in places, and an inner single layer of flattened cells, the perilemma (Hoyle, 1952). The latter forms a continuous cytoplasmic cylinder lining the whole nervous system. The tracheolated membrane probably has some effect as a diffusion barrier, especially when undamaged and receiving fresh tracheal air (see muscle section below). Most of the protection against high external potassium must, however, come from the inner layers. 112 mM. K reduces the nerve spike slowly at first, but after about 2 hr. a steady plateau is reached showing that the sheath may be doing active work. Evidence that the sheath surrounding the fine distal branches of the nerves has similar properties has been obtained and is described in the section on muscle.

MUSCLE PREPARATIONS

The simplest muscle preparations used have been recorded from intact extensor or flexor tibialis muscles using single electrodes inserted through small holes in the cuticle of the femur. Both muscles receive fast-fibre innervation from branches of the crural nerve. The threshold of the flexor fibres is lower than that of the extensor fibres, and hence 'clean' potentials can be obtained from the flexor muscles by graded stimulation of the whole crural nerve in the thorax. The stimulating and recording electrodes are placed in roughly the reverse positions to those shown in Fig. 1. Saline is admitted through a small hole bored at the proximal end of the femur and allowed to drain away through two holes bored at the distal end. These preparations are useful for some purposes, but they are complicated by the fact that each of these muscles is composed of several muscle units. The units themselves cannot be readily isolated for investigations, but use has been made of the fact that one of the muscles in the femur consists of only a single unit. This is the retractor unguis muscle which is inserted on the proximal margin of the femur. Its apodeme is 3–4 cm. long and passes down most of the femur and the whole length of the tibia and tarsus to attach to the claw of the tarsus. It is innervated by a single fast-fibre which can be stimulated by controlled stimulation of the appropriate branch of the crural nerve. The flexor and extensor tibialis muscles can be cut away almost completely, leaving this tiny muscle intact and functioning. A single stimulus via the fast axon produces a marked twitch. The effect of this is to bend the claw towards the tibia. The potentials from both types of preparation are usually monophasic, with the main spike positive with respect to earth. In some loci, however, a slight shift of the recording electrode changes the sign of the potential. Positive potentials are commonly recorded from crustacean muscles (Wiersma & Wright, 1947), and from some muscles of the cockroach (Roeder & Weiant, 1950).

Muscle preparation results

The advantage of the extensor and flexor tibialis preparations is that damage to the tracheal system can largely be avoided. With these preparations the muscle spike potentials are reduced fairly rapidly by high potassium salines (Fig. 4). Some animals appear to 'hold' 70 mM. K saline for a time, but never for more than 1–2 hr. When the spike-potential decline has started it proceeds fairly rapidly. In all instances, provided the 70 mM. K was not left for too long a period, the original potential height was restored in 5 mM. K saline. Following treatment with 140 mM. K there is an almost immediate contracture, at least of surface muscle fibres, and complete recovery of the original spike potential is not found. During the course of reduction of the extensor muscle spike potential following potassium treatment, the twitch tension which is produced by maximal single-shock stimulation of the crural nerve gradually decreases. It is quite small at 70 % original spike height, and just absent at 60 %, although the muscle still gives a small twitch to a pair of shocks at 50 msec. interval, and at 50 stimuli per sec. a vigorous contraction is present. The muscle is now entirely frequency operated. At 50 % original spike height 150 stimuli per second are needed to produce a noticeable contraction, and

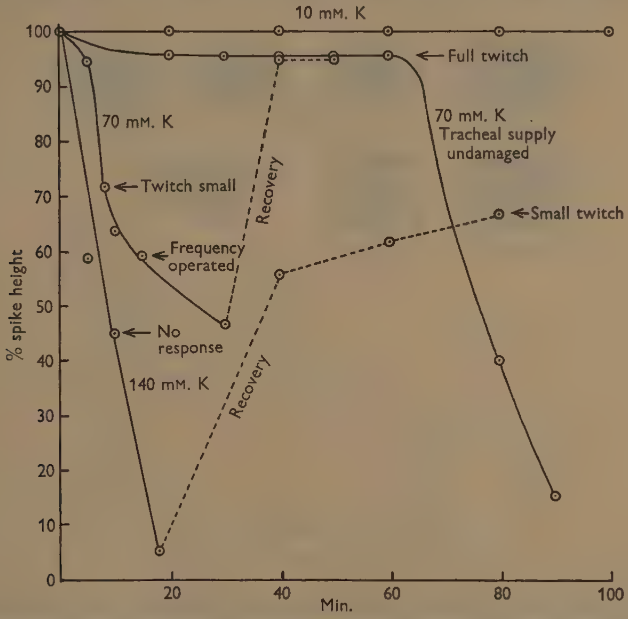

Fig. 4. The effect of high potassium concentration in the bathing fluid of the extensor tibialis on muscle spike height. The whole-muscle potential was recorded *in situ*. The dotted lines represent recovery following bathing in 10 mM. K. Four individual locusts giving typical results.

at 45 % there is no movement at all. The twitch reappears after prolonged washing with 5 mM. K saline.

Further light is thrown upon these observations by experiments with the retractor unguis muscle. Here there are only about fifteen fibres and the investing tracheolated membrane which surrounds each insect muscle unit can be torn away with glass needles without damaging the nerve. 70 mM. K saline reduces this muscle spike to a small fraction of the original potential in a few seconds. The potential is equally rapidly restored by washing the muscle in 10 mM. K saline. It is clear, then, that the muscle fibres are affected by high potassium just as rapidly as the nerve axons, and are also in line with crustacean and vertebrate muscles in this respect.

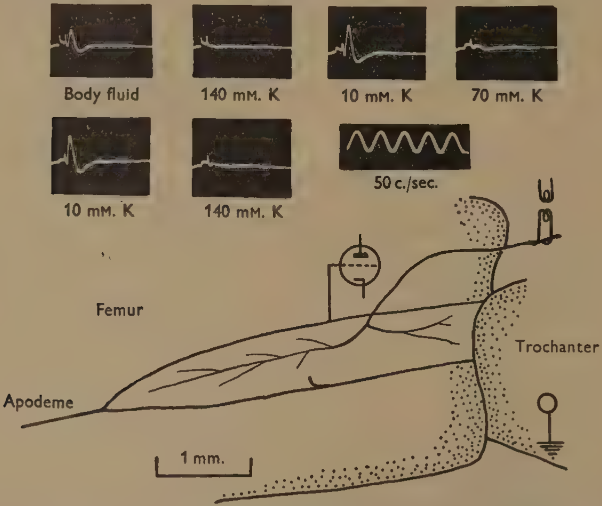

Fig. 5. The retractor unguis muscle preparation showing the effect of altering the potassium concentration of the bathing fluid successively. The intervals between each of the photographs was 1 min., during which time the bathing fluid was quickly changed. The nerve spike remained unaffected.

The slower decline of the whole intact muscle is due to two factors. First, the tracheolated investing membranes must act as partial diffusion barriers to potassium ions. Secondly, the outer fibres must prevent rapid mixing of the interstitial fluid with the externally applied saline. The retractor unguis muscle records show the nerve spike preceding the muscle spike (Fig. 5). This spike is not altered for some time after treating the preparation with high potassium saline, although the muscle spike potential is almost completely abolished. In 70 mM. K saline, which does not completely depolarize the muscle membrane, a small muscle potential is clearly present after treatment, and even in 140 mM. K some activity is apparent. It is evident, therefore, that the nerve axons are protected by the sheath even in the finer branches.

Membrane potential

Direct measurement of the muscle membrane potential has been effected with intracellular micro-electrodes using the methods of Ling & Gerard (1949) and Fatt & Katz (1951). The technique used has been to remove a metathoracic leg, mount it in plasticine with the ventral side uppermost, and remove a small piece of cuticle with underlying epidermis from the proximal end of the femur between the main cuticle ridges. This exposes several parallel fibres of a unit of the flexor tibialis muscle without the necessity of any further dissection. The whole is covered in saline and a micro-electrode thrust into a fibre. The isolated legs start to deteriorate after about 20–30 min. so experiments have been done on a number of different animals and the results averaged. The graph of resting potential against saline potassium concentration (logarithmic scale) is given in Fig. 6. Each point represents the average resting potential from several fibres of each of three or more animals. Each animal was given a preliminary 5 min. in the normal saline and then taken to the test saline for 10 min. Different animals were used for each different saline. At the higher concentrations there is sometimes a marked step of a few millivolts as one first penetrates the tracheolated membrane before entering a fibre. Surface fibres reach a steady value in a few minutes but deeper fibres take much longer. Consistent potentials greater than 70 mV. have not been recorded at the lowest potassium concentrations, although the general shape of the curve would lead one to expect them. On the whole the curve obtained resembles very closely those obtained from both nerve and muscle fibres of other animals, except that it is displaced slightly to the right along the abscissa.

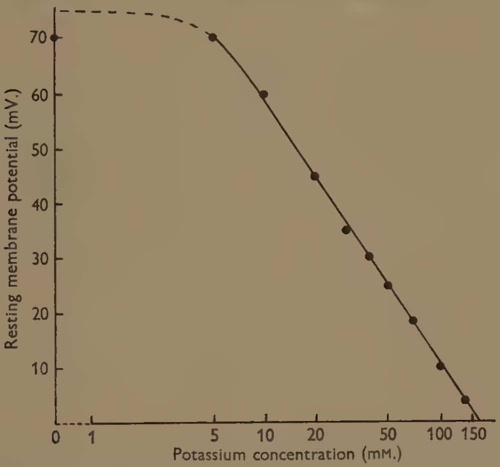

Fig. 6. The relationship between the membrane resting potential of fibres of the flexor tibialis muscle and external potassium concentration.

Absence of sodium

Sodium-free salines containing 10 mM. K and isotonic glucose to replace the sodium reduce the spike potential of the retractor unguis muscle in a few seconds. The potential was partly restored by replacing the glucose with isotonic choline chloride. Nerve stimulation then gave a prolonged muscle spike with a smaller peak potential than that obtained in normal saline.

DISCUSSION

The results of the experiments described above clearly show that the locust nerve and muscle membranes are affected by potassium ions in a similar way to the vertebrate and crustacean fibres studied by previous investigators. It is reasonable to suppose that other insects are not significantly different in this respect. Roeder (1948) found reversible blockage of conduction of *Periplaneta* nerve in 50 mM. K saline. The suggestion of Hodgkin (1951) that insects may have evolved a different method of producing nerve and muscle potentials can, in part, safely be ruled out. It is not necessary, at least, to invoke a different relationship to potassium ions. The problem of the high potassium in the haemolymph still remains. The suggestion of Heilbrunn (1943), that the potassium may be bound to organic materials and be effectively absent, may still apply. However, it is clear that even if the high potassium values reported represent free potassium ions, the herbivorous insects may be able to cope with them adequately at least for short periods, if not indefinitely. The nerve axons are all surrounded by a membrane which is partly impermeable to potassium ions and which may be capable of doing active work to provide the axons with their own stable environment independent of fluctuations in haemolymph concentration. The muscle fibres are not so well protected, although tracheolated membranes investing muscle units offer a temporary safeguard against a sudden change in blood concentration. The fibres are, nevertheless, ultimately at the mercy of blood potassium. They can, however, still produce contractions when partially depolarized by high potassium, principally because, like crustaceans (Wiersma, 1941), they do not rely on propagated action potentials for the normal operation of the contractile mechanism, but instead respond to the facilitating effect of successive impulses in a continuous series (Pringle, 1939; Roeder & Weiant, 1950).

These observations make it possible to explain the observations of Ramsay (1952), on the effect of feeding *Rhodnius* on blood containing potassium chloride. These animals survived for some hours when the haemolymph potassium was gradually raised from the normal level of about 10 mM./l. to values as high as 124 mM. following feeding on the artificial diet. These animals showed progressively weaker responses with time after feeding. As the blood concentration increased, the nerves were probably enjoying complete protection such as the sheath, which seems to be widely present in insects, could provide, as evidenced by the fact that they must still have been conducting action potentials for any muscle movements to have occurred at all. The weakened responses indicate that the muscle fibres alone were gradually being depolarized and hence capable of developing less tension, until eventually they did not respond at all.

The implications of the observations described on the effect of potassium ions on resting potential, to herbivorous insects in general, are great. The apparent blood potassium of many forms would make the muscle resting potential low, and occupy a position on the steeply sloping part of the resting potential/potassium concentration curve. Hence a change in blood potassium should tend to produce a marked change in resting potential and consequently a change in muscle properties, and perhaps also of the behaviour of the whole animal. The blood analyses described were made, as far as can be determined, on animals fed *ad lib.* on plant material. Indeed, only under such conditions can adequate haemolymph samples for analysis be obtained. Even when only partly starved, locust haemolymph becomes a thin paste which is present in surprisingly small volume. The range of qualitatively observed values of blood volume in a batch of animals reared under identical conditions is large. Potassium variations are therefore to be expected, and the values in the literature for potassium concentration may well be indications of the upper limit of these variations rather than the norm. If the mineral-regulatory machinery excretes excess potassium relatively slowly, then a heavy continuous intake of potassium by way of plant food which usually contains little sodium and much potassium (see Tobias, 1948*b*) may readily lead to an accumulation of the latter. The observed values may not indicate the 'basic' blood potassium but only that of an insect whose potassium excretory mechanism is under stress. Although attempts have been made (with success) to raise the blood potassium of omnivorous insects, I know of no experiments in which attempts have been made to lower it in herbivorous ones.

Although insects have been shown to have blood which varies widely in composition, especially when subjected to special stress, it does not follow, as Tobias (1948*a*) proposed, that the effective bathing fluid of the tissues must follow these changes. Many of the organs of insects are invested by tracheolated membranes (Wigglesworth, 1950) which may act as selective barriers between the blood and tissues. A thin film of fluid, such as must exist beneath the tracheolated membranes, containing adequate sodium and not too much potassium, is all that is necessary as a physiological medium for heart, nerve, muscle and perhaps other tissues. This may partly answer the question raised by Tobias (1948*b*), that there are only minute amounts of sodium in the pupae of *Bombyx mori*. The range of observed haemolymph potassium values described in the literature, from a variety of insects, does not include any which *Locusta* could not tolerate, at least for some time, although undoubtedly the higher values would seriously impair muscle efficiency.

SUMMARY

1. The ionic composition of the blood of insects, unlike that of vertebrates and most other invertebrates, shows a wide range of values, especially in regard to the physiologically important ions of sodium and potassium.

2. Preparations are described in which the effect of potassium on the active nerve and muscle properties of *Locusta migratoria* have been investigated.

3. The nerve axons are depolarized by high concentrations of potassium ions in the same way as vertebrate and crustacean axons.

4. The whole nervous system is surrounded by a sheath which is an effective barrier to the diffusion of potassium ions.

5. The muscle-fibre membrane is also depolarized by high concentrations of potassium ions in a similar way to muscle-fibre membranes of other animals which have been previously investigated.

6. The tracheolated membranes surrounding the muscles, and the close arrangement of the constituent fibres, delay the passage of potassium ions through the muscles.

7. It is predicted that the mechanical properties of the muscles of a herbivorous insect will be found to be subject to fairly marked changes if the blood potassium rises or falls to an appreciable extent.

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PHYSIOLOGICAL AND PHARMACOLOGICAL OBSERVATIONS ON MUSCLE RECEPTOR ORGANS OF THE CRAYFISH, *CAMBARUS CLARKII* GIRARD

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(With Plate 8)

INTRODUCTION

Alexandrowicz (1951) has recently described organs in the abdomen and thorax of the lobsters, *Homarus vulgaris* L. and *Palinurus vulgaris* L., which he considers to be stretch receptors. Their structure is reminiscent of that of the (vertebrate) muscle spindle. These organs are few in number and were found only in the dorsal regions of the body. Two pairs in each of the six abdominal segments and a small number (perhaps only four) in the thorax may constitute the animal's entire complement of these receptors.

The organs of the abdomen are attended by an interesting, if complex, innervation. One of the several axons supplying each unit has its cell body in the periphery, in close apposition to a specialized area which Alexandrowicz calls the 'intercalated tendinous region'. The cell sends short dendritic processes to this area and is considered to have a sensory function; its axon would conduct impulses centripetally in response to stretch. Also observed were fibres typical of crustacean motor axons. They branch extensively along the muscular regions of the unit, 'giving off abundant ramifications ending amidst the myofibrils'. Other fibres, which seemed to branch and innervate the tendinous region as well as the muscular areas, were described and referred to as 'accessory' nerves.

It seemed of special interest to study the physiology of these crustacean receptors in view of the physiological deductions made by Alexandrowicz and of the recent intensive investigation of the vertebrate muscle spindle, both anatomically (Barker, 1948) and physiologically (Lecksell, 1945; Katz, 1949, 1950*a*, *b*; Kuffler, Hunt & Quilliam, 1951; Hunt & Kuffler, 1951*a*, *b*; Hunt, 1952*a*).

Several attributes of these receptors contribute to making them more amenable to experimentation. Unlike the vertebrate spindle, which lies within the body of a muscle, these are separate and external organs, free from other musculature and having only nervous connexions. They are thus rather easily isolated and, if necessary, removed from the animal. Carefully dissected preparations, if perfused occasionally with fresh solution, maintain their ability to respond to stretch for long periods, often for over 12 hr.

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The present paper aims to present a first approach to the study of some of the reactions of these units. Several aspects of the present work will be treated in more detail in subsequent research, but since these organs represent one of the more easily obtained single-unit sensory preparations, and since their exposed position allows their being bathed directly in solutions of known drug or ion concentration, it has seemed appropriate at the present time to follow our short preliminary note (Wiersma, Florey & Furshpan, 1952) by a more detailed description of technique and results.

METHODS

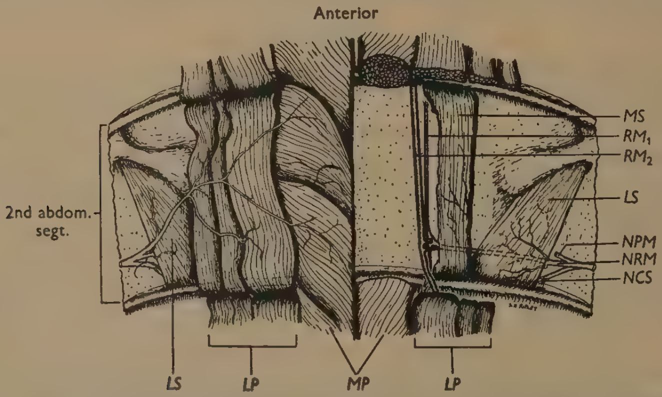
Most experiments were performed with the fresh-water crayfish, *Cambarus clarkii* Girard, and some, as noted in the text, with the lobster, *Panulirus interruptus* (Randall). Several methods of preparation have been employed. One dissection has been found best for most purposes, however, and although similar to one given by Alexandrowicz, it will be described in view of some anatomical differences between lobster and crayfish.

The tergal parts of all the abdominal segments are dissected free from the rest of the abdomen in the form of a single strip containing all the extensor musculature. It is found convenient to hold the strip, ventral surface up, with a bulldog clamp which can be attached with a spring clip to the side of the perfusing vessel. The clamp is attached to the proximal end of the first abdominal tergite. Varying amounts of the dorsal attachments of the flexor muscles remain, and these are removed from the half-segment to be prepared. After severing the nerve branch supplying it (Text-fig. 1, *NPM*), the more ventral layer of extensor musculature (profundis lateralis and medialis) is cleared, care being exercised to cut nerve branches which connect the thicker of the two receptor organs with the profundis musculature. Now the superficial extensor muscles are exposed and the receptor units can be found by carefully pulling aside the medial border of the medial superficial muscle, which partially covers the units (Text-fig. 1, right side). Removal of the tergal strip necessarily severs the nerve trunk which supplies the organs, but a sufficient length of nerve remains to allow for the placement of electrodes. If the organs are to be excised this section of nerve may be freed along its entire length by carefully removing the remaining extensor musculature to which it sends numerous small branches. If the units are to be left within the tergal strip, however, only the medial and not the lateral superficial muscle is removed, as the latter then serves to protect the units from being stretched by pull on the nerve. This same trunk also sends at least one large branch (Text-fig. 1, *NCS*) dorsad to tactile receptors in the tergum, and this branch is always cut since impulses from these receptors otherwise confuse the recording.

To test the response to stretch, the end of the cut nerve trunk was lifted above the surface of the perfusion solution on a micromanipulated platinum electrode. The other electrode was immersed in the surrounding fluid or attached to the clamp which holds the strip. Most of the recordings were taken from organs of the second segment. A thread was attached to the posterior part of the strip so that pull upon it caused movement of the third segment on its articulation with the second similar to that in natural flexion.

After suitable amplification the impulses were led into an oscilloscope, or in some experiments concerned only with low-frequency response, into a power amplifier which drove a signal magnet writing on a two-drum kymograph. With this device long continuous records have been easily and inexpensively obtained.

Preparations were dissected in, and perfused with, van Harreveld's crayfish solution (van Harreveld, 1936). Some of the preparations for testing motor response were stimulated in a solution containing one and one-half times the normal amount of potassium and no magnesium (Waterman, 1941). To study the effect of drugs on the receptors, drops of the appropriate solutions were applied to either stretched or unstretched preparations, and the concavity of the tergal segment served conveniently to retain the added solution for a short period of time. If it was desired to have the drugs act for a longer time, the entire vessel within which the strip was held was filled with the solution. Concentrated standard solutions of drugs were prepared in van Harreveld crayfish solution containing no bicarbonate, and were diluted with buffered solution prior to being used.



Text-fig. 1. Semi-diagrammatic drawing showing the location of the receptor organs, nerve trunk and branches, and the extensor musculature in the crayfish. It represents a ventral view of a section of the tergum which has been removed from the abdomen in a single strip. The letters denote the following structures. Extensor musculature: *MS*, superficialis medialis; *LS*, superficialis lateralis; *MP*, profundis medialis; *LP*, profundis lateralis. Nerve branches: *NRM*, nerve to the receptors and the superficial extensor muscles; *NPM*, nerve to the profundis muscles; and *NCS*, nerve to the various receptors of the exoskeleton. RM_1 and RM_2 are the muscle receptor organs.

Anatomy

A short comparative study of the anatomy of the crayfish and lobster organs was made. The anatomical data for the lobster are, for the most part, from Alexandrowicz; but several preparations of the California rock lobster, *Panulirus interruptus*

(Randall), were made for further comparison. In preparations dissected as described above, the two receptors are easily seen under the binocular microscope without the aid of staining. It was necessary to stain, however, in order to see the nerves and ganglion cells. For this purpose rongalite-reduced methylene blue (Pantin, 1948) was found quite satisfactory.

In the crayfish as in the lobsters, the two organs of each half-segment are of unequal length and thickness. The difference in thickness is much more pronounced in the crayfish than in the lobster, but in both the thicker organ is also the longer. As is the case in the lobster, the larger of the two organs exhibits finer cross-striation of the muscular regions. As already indicated in the section on methods, there is a surprising difference between crayfish and lobster in the position within the abdominal segment. In all of the lobsters these organs are situated in the space between the lateral and medial superficial extensor muscles. This places them lateral to the superficialis medialis, whereas the organs of Cambarus lie medial to this muscle (cf. Text-fig. 1). Another difference between the two animals exists in the amount of connective tissue surrounding the organs. Whereas in *Panulirus* the two organs of a half-segment are almost entirely enclosed within an encapsulating sheath, those of the crayfish are separate and rarely bound together by any external connective tissue except in the region of the nerve entrance.

In both animals it was possible to show the presence of ganglion cells sending dendrites to a differentiated region. Fibres typical of motor axons, whose branches ramified along the length of the muscular parts, were also seen; and in one case fibres were observed which seemed to fit Alexandrowicz's description of accessory nerves. Thus, despite the differences, the similarities seem sufficiently manifest to justify carrying over Alexandrowicz's terminology to the crayfish. The longer and thicker organ, having the finer cross-striations, will be called RM_2, and the other, RM_1.

Response of the receptors

Flexion of the tail strip is followed by trains of action spikes in the trunk innervating the *RM*'s (muscle receptors). If the precaution of severing the branch to the receptors in the exoskeleton has been taken, spikes of not more than two heights are observed, each falling into a regular rhythm (Pl. 1, fig. 1). The two rhythms are easily distinguishable from one another, since one adapts very slowly and the other by comparison, rather rapidly. A marked difference in stretch-threshold for eliciting the two different rhythms is also apparent. The slowly adapting rhythm follows much slighter stretch and, in fact, is occasionally recorded in the absence of any externally applied stretch. It is therefore easily obtainable by itself (Pl. 1, fig. 3). The regularity of this rhythm, as well as the constancy of the spike height, can leave no doubt that it arises from a single receptor unit. This slowly adapting unit can maintain continuous discharge for periods exceeding an hour, and in one experiment, where the preparation was constantly perfused at a slow rate, response to a sustained stretch did not cease until $3\frac{1}{2}$ hr. after its initiation. As intimated, the other rhythm arises from a unit which has a higher threshold and is fairly rapidly adapting. It is usually necessary to flex the isolated tergal strip to an extent which approaches the maximum possible in order to induce discharge in this receptor unit. Even on maximal flexion the response usually lasts for about 30 sec. and seldom longer than 1 min. By the standards of tactile receptors, a discharge which is maintained for a minute is long-lasting. In the crayfish, however, where this unit is contrasted with another which is capable of sustained response for several hours, it has been convenient to refer to it as the 'fast-adapting' receptor unit.

There are, then, two distinct types of rhythm which can be recorded from the nerve trunk to the *RM*'s. Histologically two types of organs, RM_1 and RM_2, have been shown to be present, and the question arises which receptor gives rise to which type of rhythm. If a moderate amount of stretch is applied to a preparation so that only the slow-adapting unit is active and then the terminal attachments of RM_2 (the longer-thicker organ) are severed, no appreciable change in the frequency of the discharge ensues. But if the attachments of RM_1 are then cut, all discharge ceases. If in other preparations, RM_2 is left intact, and only the attachments of RM_1 are cut, some response, though always diminished in frequency, may follow upon gentle stretching. This is due to connective tissue binding the two organs together in the region of the ganglion cells, and this response can be shown to originate in RM_1 despite its severed attachments. Further, it can be shown more directly, by the converse experiments, that the fast-adapting rhythm originates in the organ RM_2. It has recently been found possible completely to isolate and remove one or the other of the *RM*'s from the tergal strip, and to study it in isolation. These experiments leave no doubt concerning which type of rhythm arises from which receptor. In most preparations the nerve-action potentials recorded from RM_1 were smaller than those of RM_2 (Pl. 8, fig. 1A). In the rock lobster a similar pattern of two spike rhythms was observed. Because of the connective tissue enclosing the two receptor muscles in this animal, no cutting experiments were performed; but, as in the crayfish, the more slowly adapting discharge with the lower threshold consisted of spikes of smaller size in almost all preparations (Pl. 8, fig. 1B).

Only one of the nerve fibres supplying each receptor unit has its cell body in the periphery and is thus probably the sensory axon. Further evidence in support of this interpretation was obtained physiologically. The most direct and conclusive method would involve splitting the receptor nerve trunk and recording from single axons. Successful isolation of single axons has not yet been accomplished on account of the extremely tough connective tissue surrounding the nerve fibres in the periphery, and a less direct method had to be employed. The muscular elements of the receptor unit receive branches from all the attending nerve fibres with the exception of that one whose cell-body lies peripherally and whose terminals are restricted to the narrowly circumscribed tendinous region. It was found that crushing the muscular parts of an organ with a fine forceps did not alter the response to stretch. But if the approximate area innervated by the dendritic processes of the ganglion cell was probed with a sharp needle, a high-frequency discharge resulted and was usually followed by a loss of the receptor's ability to respond. Both of these facts are in full accord with the assumption that the fibre with the peripheral cell-body is indeed the sensory axon.

It was then found feasible to clamp the tendinous region with two pairs of screw-locking forceps, one on either side, so that very little muscle tissue was interposed. One pair of forceps was secured and the other moved by a rack and pinion device, thus stretching the receptor region almost exclusively. Responses were readily obtained in this manner, and it was found that the characteristic differences in the responses of the two receptors were not altered with this method of stretching. The comparatively rapid adaptation was still displayed by RM_2, and it is thus a property of the receptive mechanism or is an attribute of its nerve fibre (Gray & Matthews, 1951), rather than a function of its attachment or of the viscous-elastic properties of its in-series muscle elements.

These differences in the response to stretch of RM_1 and RM_2 are paralleled by disparities in the response of the receptors to various drugs and to alteration of the ionic content of the perfusing solution. Differences in the effects of drugs will be noted in a subsequent section, but some differences in the responses to changes in calcium and potassium ion will be noted here.

The increase in excitability caused by a reduction in the external calcium concentration is well known and has been specifically studied for receptors by Talaat (1933). Elimination of this ion from the external environment of these muscle receptors also produces dramatic increases in their excitability. Shortly after their immersion in a calcium-free solution, the receptors begin to fire spontaneously. If allowed to remain in this medium, the frequency of the response continuously increases until a point is reached at which impulses seem to drop out of an otherwise regular train. The dropping out continues and eventually the spontaneous activity, as well as the ability to respond to stretch, disappears. It has been consistently noted, however, that the response of RM_2 to the decreased calcium ion is delayed with respect to that of RM_1. Often impulses have started to drop out of discharge from RM_1 before RM_2 starts to fire spontaneously. Differences have also been found between the responses of the two receptors to increases in the external potassium concentration. Results with this ion were not as consistent as those obtained with calcium, but a general pattern was observable. The high-potassium solutions were made by adding crystalline potassium chloride to normal crayfish solution. Potassium concentrations of three and four times normal caused a short (*c.* 30 sec.) decrease in excitability so that a moderate discharge resulting from stretch would be inhibited. This phase was followed by one of increasing excitability, and with higher concentrations (4 ×) spontaneous activity would ensue in RM_1. The increase in excitability often continued, and a dropping out of the spontaneous impulses and a cessation of discharge similar to that described above for calcium lack would eventually occur. Such spontaneous activity, and the associated behaviour, was never seen for RM_2 with these potassium concentrations.

The dropping out of impulses followed by complete cessation of spontaneous discharge found in calcium-free solutions resembles, at least superficially, another phenomenon noted in the behaviour of RM_1. If stretch on this receptor is continuously increased, a point near the extreme limit of flexion will be reached at which impulses start to drop out. Then almost immediately, the high-frequency discharge stops completely. Following a slight decrease in tension, the discharge is again resumed. Recovery will also occur if the receptor merely remains in this over-stretched state for some time. In one such experiment the receptor was over-stretched and maintained in this condition until, at the end of approximately 4 min. discharge was resumed. Then tension on the receptor was increased further until the discharge again ceased, and again recovery occurred after 4 min. This was repeated for three additional trials with recovery times of 4, 4½ and 5 min. The tensions needed to produce over-stretch are rather high, and it is possible that these recovery effects find their explanation in the slow rupture and slipping of the in-series elements of the organs. Some of the other attributes of the over-stretch phenomenon will be mentioned, although it is difficult to evaluate their significance without further study. The frequency of discharge immediately prior to cessation in over-stretch is dependent upon the rate of stretching; the higher the rate, the higher the frequency. This frequency is also dependent upon the state of fatigue of the preparation; the less fatigued, the higher the frequency. Over-stretch occurs at lower tensions, and is preceded by lower frequencies if the external potassium concentration is higher than normal.

Motor innervation

Because of the ubiquity of multiple innervation and peripheral inhibition in crustacean motor systems, it is usually necessary to obtain single axons before an effective study of these systems can be undertaken. Numerous attempts to induce contraction in the receptor musculature by stimulating the whole nerve trunk have been made. These have been unsuccessful, with a few exceptions, despite the apparent good condition of the animals and the care with which the dissections have been executed. This lack of success may, perhaps, be attributable to the above explanation (an interaction of motor and inhibitory stimulation), but attempts to isolate single axons have also been unsuccessful, as the connective tissue band in which the axons lie is extremely tough. Nevertheless, the few preparations which have given contraction on stimulation of the nerve trunk, demonstrate the existence of a functional motor system. It has also been shown that, as in the vertebrate spindle, this motor system is capable of altering the response to a given stretch. Pl. 8, fig. 2 shows several superimposed sweeps of the oscillograph. The recording electrode was placed on the nerve not far from the stimulating electrodes, and thus the stimulus artefact and deflexion of the base-line are rather large. The impulses which were set up at the stimulating electrodes and travelled directly to the recording electrode are included in the stimulus artefact and are not seen in the picture. The musculature of both organs gave visible contractions and impulses in both sensory axons are seen in the record.

There are several other muscle systems whose contractions interact with the receptor organs and affect their sensory discharge. It is known that contraction of the flexor muscles will stretch the receptors, and it is probably this system which is capable of imposing the widest range of tensions on the organs. Contraction of the extensor muscles would also be expected to affect the receptor's discharge, and the following experiments were performed to see if these expected effects were present. A tergal strip was removed as usual, the remnants of flexor musculature cleared, but no further dissection was performed. Then the nerve trunk of the right half of the second segment was picked up on a recording electrode and that of the left side of the same segment on two stimulating electrodes. A base-line discharge was set up by moderate flexion of the strip and then the stimulating electrodes were activated at a frequency of about 30/sec. A visible contraction of the extensors of the left side was accompanied by a decrease or cessation of the discharge from the receptors of the right side. It is presumed that contraction of the extensors of the same side of the same segment would inhibit discharge of the receptors even more readily. Experiments performed in a similar manner demonstrated that contraction of the extensor muscles of the right side of the third segment had an opposite effect and augmented discharge from receptors of the right side of the second segment. It can be seen that very complex interactions of at least four different muscle groups are possible, all of which affect the tension on the receptor terminals, and these are considered further in the discussion.

Pharmacological reactions of the receptors

Drops of the appropriate solutions were added to the half-segment of the tergum containing the dissected receptors. At the end of 3 sec., in most experiments, the response was recorded. After the record was taken the tergal strip was thoroughly washed and then the perfusion vessel allowed to drain in preparation for addition of the next test solution. The mechanical agitation caused by the application of drops of the drugs was frequently sufficient to induce a small and transitory discharge in the more sensitive RM_1. Most of the effects with which we have been concerned, however, have been so large and long-lasting that confusion could seldom arise between mechanically caused and drug-induced responses. Where doubt existed drops of physiological solution, applied in the same manner, were used as controls.

A large number of drugs have been tested on this preparation. Of these acetylcholine gave the most interesting results, and only the effects of this drug and of a few other drugs which seemed most closely bound up with the ACh-induced response will be reported here. All drug concentrations are given in grams per millilitre of solution.

Acetylcholine. When applied in concentrations of 10^{-6} or greater, ACh consistently induces spontaneous impulses in RM_1, the slowly adapting receptor. RM_2 has about a hundred-fold lower sensitivity to ACh. In a number of fresh preparations solutions of 10^{-9} (and, in a few, 10^{-10}) ACh initially enhanced the discharge from slightly stretched preparations (that is, with a background discharge of about 10/sec.) as shown in Pl. 8, fig. 3. Subsequent applications of the same or even higher concentrations (10^{-8} and 10^{-7}) often gave no increment. Because of this lack of correlation between concentration and response the results obtained with these low concentrations are rather difficult to interpret. The frequency of discharge following application of the higher concentrations (10^{-6} to 10^{-3}) was always related to concentration, and a typical record is shown in Pl. 8, fig. 4A. The numbers inserted refer to the concentration of ACh added. The variation in height of the nerve-action potentials from one record to another is not significant but merely reflects slight differences in the level of the surface of the solution in the tergal concavity and thus in the point at which the active lead from the nerve is located. These responses were obtained from a preparation of RM_1 with intact terminal attachments but to which no external stretch had been applied.

Hunt (1952*b*) reports that ACh exhibits a large excitatory effect on the vertebrate spindle, but he concludes that it is the contraction of the intrafusal muscle fibres which causes the heightened response rather than any direct effect of the ACh on the sensory terminals themselves. Two types of experiment indicate that this is not the case, however, in the crayfish organs. In the first, the terminal attachments of the receptor were severed so that it floated loose in the ACh solution being tested, attached only by its nerve to the tergal strip. This usually resulted in only a slight decrease in the ACh-sensitivity of the preparation. For example, in one experiment an eserinized receptor (RM_1) gave a slight response to 10^{-8} ACh prior to cutting its ends free from its attachments. After this operation ACh in a concentration of 5×10^{-8} was needed to give a similar response. It is felt that this slight decrease in sensitivity to ACh reflects the decrease in the level of excitability following removal of the subliminal stretch to which the organ is subject when *in situ*. This is corroborated by a number of preparations which have discharged spontaneously at a low level without the application of external stretch. The majority of preparations of RM_1, in fact, are very close to firing, even though the tergal strip is fully extended, as only the slightest amount of flexion is necessary to initiate discharge. A second type of experiment also indicates that ACh does not produce its excitatory effect on this organ by first initiating contraction of the receptor musculature. RM_1 was completely isolated with the nerve trunk and clamped with forceps (see p. 141) on either side of the intercalated tendinous region. Fixed in this manner there is only a small amount of muscle tissue remaining in series with the receptor region, and this must certainly be damaged by the clamping procedure. But even when so clamped, the sensitivity of RM_1 to ACh was essentially unchanged.

Eserine. Despite negative effects obtained in a few preliminary experiments (Wiersma *et al.* 1952), eserine (physostigmine salicylate Merck) has since been found to enhance the ACh response of all preparations tested, to a rather marked extent, when they are bathed in this drug for some time (3–5 min.). It has recently been found that high concentrations of eserine can also show a depressant action on ACh excitation. If drops of 10^{-4} eserine are followed immediately by ACh, a much smaller excitatory effect is obtained than if a washing is interposed between the applications of the two drugs. We have not studied this effect further, since it is not directly concerned with the problem in hand, but it might be that in this direction lies an explanation of the preliminary negative results.

Pl. 8, fig. 4E shows some responses of the same preparation of RM_1 as is shown in Pl. 8, fig. 4A, but between the times of the two sets of recordings the receptor had been bathed in eserine 10^{-5} for 3 min. No external stretch was applied, and the numbers inserted again refer to the concentration of the single drops of ACh which were added 3 sec. prior to the taking of each record. It can be seen that there is an approximately ten-fold increase in the sensitivity of the eserinized receptor to ACh. Similar results have now been obtained in a large number of experiments. After being bathed in the lower concentrations of eserine (10^{-5}, 10^{-6}), the receptor shows no noticeable change in its sensitivity to stretch stimuli. Higher concentrations (10^{-4}), however, usually induce a low-frequency discharge in the absence of externally applied stretch. Once obtained, this spontaneous discharge is difficult to suppress and may persist after a number of washings.

Atropine. Many drugs, when applied to the receptors in high concentrations, induce effects which may not be related to their more typical and specific actions. In fact, one is hard pressed to find a compound which in high concentrations does not have some effect on the response of this naked receptor; and, especially in the invertebrates whose pharmacology is less well defined than that of the vertebrates, it is difficult to decide where the boundaries between 'typical' and 'unspecific' effects should be drawn. This difficulty is well illustrated by the effects of atropine. When this drug is applied in a concentration of 10^{-4}, it causes considerable augmentation of response in discharging receptors (Pl. 8, fig. 5*c*, *d*), and can initiate spontaneous activity in resting ones. Atropine 10^{-5} also has a slight excitatory effect. But more striking is the effect of the atropine on the ACh-induced excitation. Pl. 8, fig. 5*a* shows the response of a preparation of RM_1 to a slight stretch. Pl. 8, fig. 5*b* shows the response of this same receptor three seconds after the addition of one drop of ACh 10^{-4}. After washing for a short time the discharge (Pl. 8, fig. 5*c*) returns to its original level. Then atropine 10^{-4} is added and the excitation which it induces is shown in record 4*d*. Subsequent addition of another drop of ACh 10^{-4} causes only the slight increment of discharge (over that induced by atropine) seen in Pl. 8, fig. 4E. After washing ACh 10^{-4} is again capable of inducing the same large response (Pl. 8, fig. 5*f*) as prior to the administration of atropine. These effects of atropine were consistently observed in a large number of preparations, and in no case did it cause a depression of the normal stretch response.

DISCUSSION

These organs have thus proved to be receptors responding to the stimulus of stretch. This fact and several other facts were correctly predicted by Alexandrowicz. He also foresaw that the fibres ramifying along the receptor musculature subserved a motor function, and that the receptor designated as RM_2 would have the higher threshold. Not only was the predicted difference in threshold found, but further, the two receptors were seen to have markedly different adaptation rates. RM_1 adapts very slowly, has a very low threshold, and in some instances discharges even when the tergal strip is fully extended. It can maintain both very low (about 2/sec.) and fairly high (over 200/sec.) frequencies of discharge and can do so for long periods of time. RM_1 may thus be termed a tonic receptor, for it is well suited to transmit continuous information about the degree of flexion of each segment of the abdomen. RM_2, on the other hand, fits the definition of a phasic receptor. Its high threshold and fairly rapid adaptation restricts its response, so that in the whole animal it probably fires only during rather extreme tail flexion as is found in the swimming reflex. Alexandrowicz also felt that it would be involved in this reflex, and further conjectured that RM_2 might initiate impulses which inhibit the powerful tail flexors. It seems to us more likely that impulses from this receptor operate to induce reflex contraction of the extensors so that the tail is readied for its next flexion.

A knowledge of all the functional connexions of the motor nerve fibres innervating the receptors will certainly facilitate the formulation of the role of the receptors in reflexes, in view of the complicated interactions which could conceivably exist in such a system. Contraction of the *RM*'s has been shown to facilitate discharge; but if these organs have been phylogenetically derived from the extensor muscles and share with them one or more motor axons, it would be difficult to state, *a priori*, how the combination of in-parallel extensor-muscle contraction and in-series receptor-muscle contraction might affect the discharge. There is, in addition the possibility of interaction between the receptors of one segment with the muscles of adjacent segments. Both of the receptors have one attachment on an articular membrane which connects tergites of adjoining segments (see Text-fig. 1), and RM_2 has its other attachment at the anterior tendon of the medial superficial muscle of the immediately posterior segment. Thus contraction of the extensor muscles of adjacent segments would be expected to increase discharge from the receptors, and this was demonstrated experimentally. Briefly reviewing the possible interactions of the abdominal receptors with other musculature of the tail, it can be seen that contraction of the flexor muscles will tend to increase discharge from these receptors. Contraction of the in-series muscle elements of the receptors themselves can also increase discharge frequency, whereas contraction of the extensor muscles of either side of the same segment will diminish the response. And lastly, contraction of the extensor musculature of adjoining segments will augment discharge. Deduction of the pattern of the discharge for any natural movement of the tail cannot be made until it is known what combination of these various motor systems may be activated simultaneously.

It is interesting to note that the difference in response of RM_1 and RM_2 to various conditions is not solely a function of the viscous-elastic properties of their in-series elements or the places of their terminal attachment parts. There must be in addition an inherent difference in their sensory mechanisms, as shown by their reactions to drugs and by the reactions to stretch of their isolated intercalated areas held in forceps. At present we are not yet able to judge the relative importance of these factors in the overall responses obtained from the organs *in situ*.

A comparison between these organs and the spindle of the vertebrates suggests itself. Alexandrowicz has already undertaken such a discussion, but since the appearance of his paper the work of Kuffler *et al.* (1951), and Hunt & Kuffler (1951*a*, *b*) on the function of the small nerve fibres in the ventral roots of cats has been published. These investigations suggest the nature of the augmentation of afferent discharge upon small-nerve stimulation first observed by Leksell (1945). Their evidence indicates that the small-nerve fibres are motor axons for the intrafusal muscle fibres, and it is by contraction of this spindle musculature that the increase in discharge is effected. A similar mechanism is now seen to exist in the crustacean muscle receptors. Although it has not been possible to split the main nerve trunk, it has been demonstrated that when stimulation of the whole nerve results in visible contraction of the receptor muscle elements, one or several impulses can be recorded from the sensory axons following each stimulus with a delay of about 10 msec. Leksell discusses the possibility of a direct facilitation of the sensory mechanism by a depolarization induced by the efferent impulses. Although it now seems that this is not the case in the spindle, the possibility of such an additional facilitating mechanism in the *RM*'s cannot yet be excluded. This, as suggested by Alexandrowicz, might be the function of the axons that he has named the 'accessory' fibres, but no evidence concerning this point has yet been found.

With respect to the response of these organs to ACh, several previous reports of a sensitivity of afferent endings to this drug are in the literature. Coon & Rothman (1939*a*, *b*) found that ACh, in an optimal concentration of 1:40,000 (as well as nicotine, 1:100,000 and α-lobeline, 1:1,000,000), can initiate axon reflexes, causing transient pilomotor and sweat responses. Concentrations higher than optimal of ACh or nicotine only inhibit further response to the optimal concentration, and the pilomotor response, at least, is not blocked by atropine. ACh can, therefore, be said to exhibit nicotine-like effects. Brown & Gray (1948) have demonstrated a similar nicotinic action of ACh in initiating spontaneous activity in nerves of the mesentery of the cat and the skin of the dog. By the elimination of other known possibilities they deduced that the recorded response must have been set up in the sensory endings by a direct action of ACh at these sites. Here, as in the pilomotor axon-reflex responses, atropine had no blocking action, and inhibition was obtainable by prior administration of higher doses of the ACh (or nicotine). This inhibition of the ACh response was not accompanied by any apparent loss of sensitivity to the stimulus of pressure and they concluded that ACh is not involved in the normal sensory function of the receptors concerned. Hellauer (1950) found that 1 % ACh solutions, dropped on the cornea of the eye, caused sensations of pain; and Umrath (1951) reports that in sensitive persons, ACh 10^{-5} can be felt as pain.

With the crayfish muscle receptor, RM_1, however, it has been possible to demonstrate directly that ACh, in low concentrations, can initiate discharge in some part of a receptor mechanism. It has been shown that ACh 10^{-6} is usually a sufficient concentration to initiate spontaneous activity when applied to the isolated receptor region of RM_1. If this preparation has been previously sensitized with eserine, ACh in concentrations as low as 10^{-8} is able to produce a definite response. Not enough is known of the pharmacology of the Crustacea to assess the validity of a distinction between nicotinic and muscarinic actions of ACh in the crayfish. It was observed, however, that the ACh response could never be blocked by increasing the concentration of this drug; atropine was seen to be an effective inhibitor of the ACh excitation; and a marked potentiation was obtained by pre-treatment with eserine. If it cannot be concluded with certainty that a muscarinic action of ACh is evident here, it is at least noteworthy that the characteristics of the response differ in these respects from those previously reported for vertebrate receptors (see above).

Although atropine was able to inhibit the ACh excitation, it demonstrated no such action against the normal response of the receptor to stretch, but, in fact, its application was usually followed by an augmentation of the discharge. In order to maintain the hypothesis that ACh acts here as a normal transmitter substance, it is necessary to invoke some mechanism like the following. The terminal surfaces of the ganglion cell dendrites would be enclosed in compartments within which the ACh would be released during stretch. Neither the added ACh nor atropine is able to penetrate the pockets, and these drugs have their entire action on the cell or dendrite surfaces external to the compartments. There is, as yet, no histological evidence for the separation of the terminal dendrite surfaces from the rest of the neuron. If one is dissatisfied with the construction of such an hypothesis, one is left with at least two possible alternatives. The first is that ACh is not in any way involved in the normal mechanism of these crayfish muscle organs and their response to it is merely fortuitous. The second takes into account the sensitivity of the receptor to such low concentrations of ACh and the potentiation of this effect by eserine. ACh would have a role in the normal receptor mechanism as a regulator of the afferent discharge, rather than being the direct cause of its initiation. This latter hypothesis has the advantage of making compatible all the observed effects of the various drugs; but, as in the case of the others, no direct evidence can be brought to its support at present.

SUMMARY

1. The muscle organs recently described by Alexandrowicz in the tails of *Homarus vulgaris* and *Palinurus vulgaris*, have also been found to be present in the crayfish (*Cambarus clarkii*) and the rock lobster (*Panulirus interruptus*), and a study of them in the latter animals has been undertaken.

2. The position of these units within the abdomen of the crayfish differs from their position in the lobsters. In almost all other respects, however, there is substantial correspondence in their morphological features.

3. The organs are easily isolated and it is found that stretching them is accompanied by a discharge in one of the axons supplying each unit. There are two receptors in each half-segment of the abdomen and their responses show considerable differences. One has a very low threshold and can be sustained in continuous discharge for several hours. The threshold of the other is high and this receptor usually adapts completely to the most extreme stretch in less than 1 min.

4. A phenomenon which is designated 'over-stretch' has been observed. All-but-maximal stretch causes a reversible cessation of the previous high-frequency discharge. The discharge can be restored by slight release of tension, but even if the tension producing 'over-stretch' is maintained for a sufficient length of time, discharge will resume spontaneously. The possibility that this spontaneous return is due to a slow rupture of the receptor tissues is not excluded.

5. The muscular regions of the organs are not necessary for their sensory function.

6. It has been possible to show that stimulation of the nerve trunk can induce contraction in these muscular regions and that such contraction results in discharge in the sensory axons.

7. The unit with the low stretch-threshold responds to low concentrations of ACh and this effect is potentiated by eserine. In concentrations above 10^{-6} before eserinization and above 10^{-8} after eserinization, ACh consistently initiates rhythmic responses in relaxed units and augments the discharge from those under tension.

8. ACh affects the sensory mechanism directly; the muscular regions of the organs are not necessary for its action.

9. Atropine inhibits the ACh excitation, but only increases the response of the receptors to stretch. Eserine, except in high concentrations, has no consistent effect on the normal stretch discharge.

10. The possibility that ACh has a role in the normal receptor mechanism is discussed, but the question is left open.

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EXPLANATION OF PLATE 8

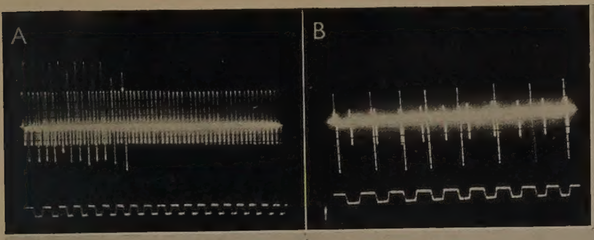
Fig. 1. Action potentials recorded from the nerve to the muscle receptors of A, the crayfish (*Cambarus clarkii*) and B, the lobster (*Panulirus interruptus*). A moderate amount of stretch has been applied in each case. In both A and B the larger spikes are from RM_2, the more rapidly adapting organ. Time signal: 60 cyc./sec.

Fig. 2. Response of sensory fibres to motor stimulation of the nerve trunk. One unit responds to each stimulus with a repetitive discharge, whereas the other responds only once. The direct response of the whole nerve trunk to each stimulus is hidden in the stimulus artefact. Time: 1000 cyc./sec.

Fig. 3. The effect of ACh 10^{-9}. A, the response of a slightly stretched preparation of RM_1; B, the increase in this response following addition of one drop of ACh 10^{-9}; and C, the return to the original discharge frequency following washing. This response to very low concentrations was not always reproducible nor related to concentration; but see fig. 4. Time: 60 cyc./sec.

Fig. 4. Response of RM_1 to various concentrations of ACh. A, before bathing in eserine 10^{-5} for 3 min., and E, after. The numbers refer to the concentration in grams/millilitre of the drop of applied ACh solution. The tergal strip was fully extended and there was no stretch-discharge prior to addition of drugs. Time signal: 60 cyc./sec.

Fig. 5. The effects of atropine. A preparation of RM_1, slightly stretched and giving the response shown in *a*. This stretch is maintained throughout the experiment. *b* shows the result of adding one drop of ACh 10^{-4}. After washing, the discharge returns to its original level, *c*. Then a drop of atropine 10^{-4} is added and a slight excitation follows, *d*. A second reapplication of a drop of ACh 10^{-4} now increases the response only slightly, as shown in *e*. Washing re-establishes the ACh sensitivity as shown in *f*. Time: 60 cyc./sec.



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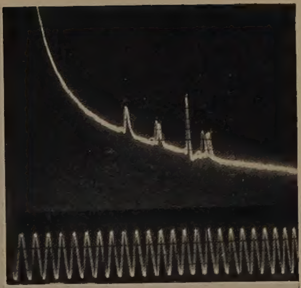

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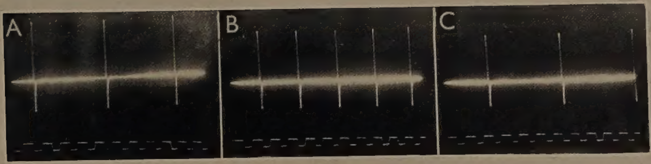

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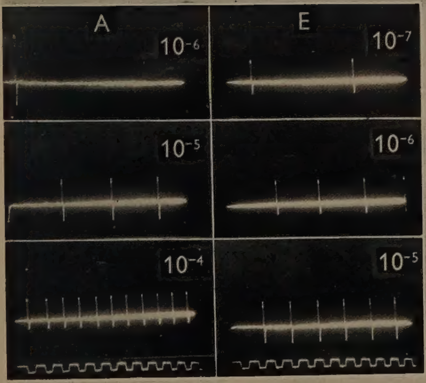

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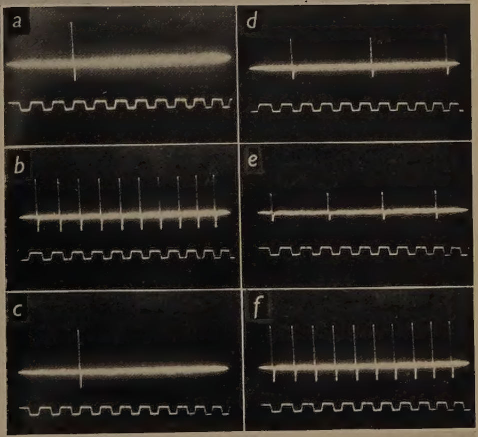

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WIERSMA, FURSHPAN AND FLOREY—MUSCLE RECEPTOR ORGANS OF THE CRAYFISH